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A STATISTICAL TEST OF THE SPECIES CONCEPT IN LITTORINA

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"One may believe that if larger series were more often utilised in taxonomic work the current bewilderment over variation would give way to a renewed respect for a certain uniformity that exists thruout such groups of individuals."—Kinsey.[1]

With certain exceptions, no two individuals of a species are ever genetically identical; hence it is not so much the uniformity as the character and range of variation in a species that are diagnostic. Conversely, the fact that two related animals differ does not necessarily mean that they belong to separate species unless it can be shown, after the examination of sufficient numbers collected over a wide area, that there is not a series of overlapping intergrades between the two differing forms.

It was, for example, the range in variation in the number of vertebræ in conger eels that enabled Johannes Schmidt (1931) to separate the American species, *Conger oceanicus*, from the European *C. vulgaris*. The ranges of the larvæ of the two species overlap in part geographically, but not anatomically, the number of vertebræ in *C. oceanicus* being from 140 to 149, average 144.63, and in *C. vulgaris* from 154 to 163, average 158.16. This lack of overlap in the numbers of their vertebræ clearly justifies their segregation.

Kinsey (1930) has shown, too, that the highly variable Gall Wasp, *Cynips erinaceus*, is one species, though extreme forms of its gall have been previously assigned to separate species. In any part of its wide range a comprehensive collection over a square mile will very closely resemble a similar comprehensive collection at any other place in the insect's range. The variation in the species is roughly constant throughout its entire geographic range.

These are two extreme cases; the first of two distinct, the second of one homogeneous, species. If, however, the two conger eels had

numbers of vertebræ ranging from 140 to 152 and from 150 to 163 respectively, it would be impossible from the vertebræ alone to assign to either group those possessing 150, 151 or 152 vertebræ. To get round this difficulty, one man would consider them mere races, another sub-species, and a third separate species with a certain amount of hybridization, the choice depending on the taxonomic upbringing and prejudices of the worker.

Again, if in such a case as *Cynips erinaceus* the amount of variation were not constant, a comprehensive collection from one part of its range would be different from a similar collection from another part. If the variations in these two collections did not overlap, they might be considered separate species unless they were merely the ends of a continuous series.

This last is the case with *Littorina obtusata*. There has been but little uniformity in the treatment of its varying forms, and the resulting taxonomic muddle must be first of all cleared up.

The establishment of the genus *Littorina* has always been ascribed to the elder Férussac, the reference in the Index Animalium (Sherborn, 1927) being to page xxxiv of the Tableaux systématiques généraux de l'embranchement des Mollusques (Férussac, 1822). This refers, however, only to where *Littorina* is listed as the fifth sub-genus of the genus "PALUDINE, Paludina, FÉRUSS. (Fluv. et marin.)," with no definition or description. The definition, such as it is, is given on page xi, where Férussac says: "Quant aux paludines marines qui constituent le genre Trochus d'Adanson, comme nous n'avons pu adopter cette dénomination à cause des *trochus* de Linné (en general formés d'espèces réellement congénères, ce qui nous a empêché de les appeler *turbo* avec Adanson, en y rapportant tous les *turbo* de Linné qui doivent s'en rapprocher), nous en formons un sous-genre sous le nom de *littorine*." (No Latin is used here, but an objection on that score would, I think, be oversteeped in pedantry, since only the final *e* needs altering.) Two pages before, at the bottom of page ix, Férussac gives a list "dressé d'aprés l'édition de Gmelin," referring to the thirteenth edition of the Systema Naturæ, edited by J. F. Gmelin (Linnæus, 1788). This list, compiled from the Linnæan genera *Trochus* and *Turbo*, which Férussac wished to combine, includes five species referred to as "*Paludina* Marine." These are *obtusatus*, *neritoides*, *littoreus*, *muricatus*, and *afer*, constituting his sub-genus *Littorina*.

"Le genre Trochus d'Adanson," mentioned above, consists of four species described and illustrated in the Histoire Naturelle du Sénégal (Adanson, 1757). Their names, on a binomial system of Adanson's

own devising, antedate the tenth edition of the Systema Naturæ (Linnæus, 1758) by one year, and therefore do not stand. These species are beautifully illustrated in Adanson's work, and undoubtedly belong to the genus *Littorina*.

Menke, 1828, was responsible for the spellings *Litorina* and *litorea*, although both Linnæus and Férussac used two *t*'s. Menke said that the alteration made for correct Latin, which is true, but it also led to a small confusion in nomenclature which has lasted till this day. In truth, "optima nomina quæ nihil significant."

The first full definition or description of the genus *Littorina* is given in the second edition of Lamarck's Histoire Naturelle des Animaux sans Vertèbres, Vol. XI, pp. 201 et seq. (Lamarck, 1843).

Of the British Littorinas, *L. littorea* and *L. neritoides* are both described under the genus *Turbo* in the tenth (1758) edition of Linnæus' Systema Naturæ, and they have retained their original specific names and authority, except in the work of a few authors who have fortunately caused no lasting confusion, save for the fashion, introduced by Menke (1828), of spelling with one *t*.

The nomenclature of the mollusks generally known by naturalists as *L. obtusata* and *L. rudis*, however, has for a long time been a field for error and dispute.

Littorina rudis is assigned by the Marine Biological Association (1931) to Maton, quoting Jeffreys (1865). Johnson (1915) gives *L. rudis* (Donovan), quoting Gould (1870) and Donovan (1804) (whose date is erroneously given in Gould as 1800, the date of publication of the second volume; the first volume was actually published last, in 1804, after the fifth in 1803). Kuester (1856) calls the unfortunate animal *L. rudis* (Montagu), quoting Montagu (1803) and Maton and Rackett (1807), in spite of the fact that the latter authorities cite Maton (1797) as the originator of the name and description; Jeffreys (1865) also gives Maton. Forbes and Hanley (1853), however, mention Donovan (1804), though Maton and Rackett (1807) give Maton (1797), Montagu (1803) and Donovan (1804) in order of priority. Montagu disturbs this order by quoting from the then unpublished volume of Donovan, having presumably seen the manuscript or proofs, but Donovan nevertheless gives the credit of the name *rudis* to Maton (1797). Menke (1830), the first to put *Turbo rudis* into the genus *Littorina*, cites Montagu (1803) as author of the species.

Under the Law of Priority, the name *rudis* must be referred to Maton (1797), since there is no doubt of the identity of the form he described.

However, *rudis* is not the correct specific cognomen of the snail which usually passes under that name. In 1792 Olivi published a description and rough figures of a shell near Venice which he called *Turbo saxatilis*. Jeffreys (1865) states that this form is identical with *Littorina neritoides* (L.), but Dautzenburg and Fischer (1912) present on two plates sixty-two exquisitely colored and enlarged figures of *L. saxatilis* and *L. rudis*, which show that *L. rudis* is specifically indistinguishable from *L. saxatilis*, of which names the latter has the priority by five years (1792 and 1797).[1]

In the case of the snail variously known as *Littorina obtusata*, *L. littoralis* and *L. palliata*, the difficulties begin with the tenth edition of the Systema, where two very similar shells are described under different genera, to wit, *Turbo obtusatus* (Vol. I, p. 761) and *Nerita littoralis* (Vol. I, p. 777). The early British conchologists Montagu (1803), Donovan (1804) and Maton and Rackett (1807) all accepted *Nerita littoralis* as representing the common form of the English Channel and British Coasts. Here Montagu introduces a minor confusion by claiming that *Nerita littoralis* L. is the same as *Turbo neritoides* L. He is alone in this opinion, and it is difficult to see what led him to form it. In 1822 Thomas Say described *Turbo palliatus* from the coast of Maine, and this is considered by both Dautzenburg and Fischer (1915) and Johnson (1915) to be a variety or sub-species of the *Turbo obtusatus* of Linnæus.

Jeffreys (1865) gave the specific name *obtusata* to the form on the shores of the English Channel, evidently assuming the identity of the two Linnæan shells. Dautzenburg and Fischer (1915) regard the English Channel (*littoralis*), the Norwegian (*obtusata*) and the New England (*palliata*) forms as varieties or subspecies of the one species, of which the prior name, by pagination in the Systema Naturæ, must be *Littorina obtusata*. Winckworth (1922), however, assigning the British Littorinas to four genera,[2] *Littorina* Férussac, *Littorivaga* Dall, *Melarhaphe* Menke and *Neritoides* Brown, includes in the last-named the three species *obtusata* (L.), *æstuarii* (Jeffreys) and *littoralis* (L.), the existence of *obtusata* on the shores of Great Britain being thought

[1] *L. saxatilis* (Olivi) is not to be confused with *L. saxatilis* (Johnston), which is a so-called variety from Berwick, England (Jeffreys, 1865).

[2] These generic names are regarded in this paper as only of subgeneric rank. They are extremely valuable in the finer delimitation of the genus *Littorina* as a whole, but considered as genera they only bewilder the poor naturalist and field worker.

Very full lists of synomymies are given for the *obtusata-littoralis-palliata* group by Dautzenburg and Fischer (1915) and for *saxatilis-rudis* by Dautzenburg and Fischer (1912).

doubtful, all of which leads us to the impasse of a difference of opinion among experts.

This confusion is due solely to the fact that inferences about varieties have been drawn from individual specimens, and the only way to unravel this tangle is to examine a large enough number of animals until the answer to the problem ceases to be a matter of opinion.

Specimens of *L. obtusata* were therefore obtained from eleven different localities, on both sides of the Atlantic Ocean. The minimum number aimed at was one hundred from each locality, but sufficient

TABLE I

Localities, number of individuals and catalogue numbers of eleven lots of L. obtusata.
M.C.Z. = Museum of Comparative Zoology, Harvard University.
B.S.N.H. = Museum of Boston Society of Natural History.

	Locality	No.	M.C.Z.	B.S.N.H.
a..	Bergen, Norway	100	47508	
b..	Cattewater, Plymouth, England	100	76980	
c..	Church Reef, near Plymouth, England	100	76978	
d..	Near Westerly, Rhode Island	100	13980	
e..	South Cohasset, Massachusetts	100		26655
f..	Briar Neck, Gloucester, Massachusetts	86		24787
g..	Rye Beach, New Hampshire	75		26806
h..	Broad Cove, Georges River, Cushing, Maine	38	67775	
i..	Port Clyde, Knox County, Maine	74	67773	
j..	Tenants Harbour, Knox County, Maine	60	67774	
k..	Isle au Haut, Maine	100	13972	
	Total	933		

specimens were not always forthcoming. In only one case, however, was the number less than sixty. Table I gives the localities of the lots, the number of individuals, and the catalogue numbers in either the Museum of Comparative Zoölogy, Harvard University, or the Museum of the Boston Society of Natural History.

The positions of the localities are shown in the maps, Figs. 1 and 2.

The lot *a* in Table I were kindly sent to me by Professor Brinkmann of the Bergens Museum, lots *b* and *c* by the Marine Biological Association at Plymouth, England, lots *i* and *j* by Mr. N. W. Lermond, and lots *e*, *f* and *g* were lent by Mr. C. W. Johnson of the Boston Society of Natural History. The rest, *d*, *h*, and *k*, were already in the collection of Harvard University.

Each shell was measured with callipers to the nearest tenth of a millimeter along three dimensions; the length (*a*) of the final whorl

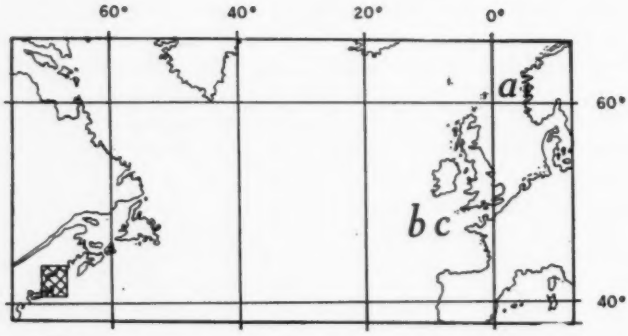

FIG. 1. Map showing the localities on the two sides of the North Atlantic. The shaded area is that shown on a larger scale in Fig. 2. The letters refer to Table I.

FIG. 2. Map showing the localities on the New England Coast. The letters refer to Table I.

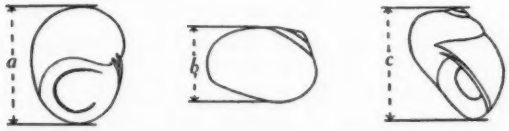

FIG. 3. Shell from Church Reef, near Plymouth, England, showing the dimensions that were measured.

(usually the overall length of the shell), the breadth (b) of the final whorl at the base of the penultimate whorl, and the distance (c) from the top of the spire to the most distant point on the lip. The ratios between these dimensions, $\frac{a}{b}$, $\frac{a}{c}$ and $\frac{c}{b}$, were calculated, and they form the basis of comparison between shells from different localities. Figure 3 shows exactly which dimensions were measured on each shell.

TABLE II

Shells from the two sides of the Atlantic: numbers of shells of different sizes.
(See Fig. 4.)

Localities	Bergen			Cattewater			Church Reef			Westerly		
Number of Shells	100			100			100			100		
Millimeters	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
5.75 to 6.25.....					1							
6.25 to 6.75.....					9							
6.75 to 7.25.....					28						1	
7.25 to 7.75.....					34	1					16	
7.75 to 8.25.....					13	7					43	
8.25 to 8.75.....		2			13	21					29	
8.75 to 9.25.....		25		7	2	30		5			10	
9.25 to 9.75.....		30		17		21		30			1	1
9.75 to 10.25.....		39		21	1	12		31				6
10.25 to 10.75.....		4	1	26		4		29				33
10.75 to 11.25.....			5	14		2		5		7		27
11.25 to 11.75.....			11	9		2			1	29		18
11.75 to 12.25.....	2		21	4					1	28		9
12.25 to 12.75.....	8		35	2					8	19		5
12.75 to 13.25.....	25		19				1		15	9		1
13.25 to 13.75.....	37		7				2		17	8		
13.75 to 14.25.....	19						17		33			
14.25 to 14.75.....	8		1				22		14			
14.75 to 15.25.....	1						28		9			
15.25 to 15.75.....							20		1			
15.75 to 16.25.....							7					
16.25 to 16.75.....							2					
16.75 to 17.25.....							1		1			

TABLE III

Shells from the two sides of the Atlantic: numbers of shells of different proportions. (See Fig. 5.)

Localities	Bergen			Cattewater			Church Reef			Westerly		
Number of Shells	100			100			100			100		
Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.99 to 1.03.....		4			1			3			1	
1.03 to 1.07.....		20			4			35			15	
1.07 to 1.11.....		52			17	1		47			53	
1.11 to 1.15.....		20			51	3		12			27	
1.15 to 1.19.....		4	1		21	18		3			4	
1.19 to 1.23.....			7		5	45			1			
1.23 to 1.27.....			29	1		22			2			5
1.27 to 1.31.....	1		33		1	7			5			12
1.31 to 1.35.....	7		18	15		1			21			32
1.35 to 1.39.....	38		10	45		2	3		22	1		28
1.39 to 1.43.....	27			26			6		34	13		17
1.43 to 1.47.....	21		2	12		1	26		7	24		5
1.47 to 1.51.....	5			1			31		7	36		
1.51 to 1.55.....							23		1	17		1
1.55 to 1.59.....	1						7			7		
1.59 to 1.63.....							4			1		
1.63 to 1.67.....										1		

It represents a shell from Church Reef, Wembury Bay, and the measurements in millimeters and the proportions calculated therefrom are as follows:

Length (a)	Breadth (b)	Spire to Lip (c)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
16.4	10.8	15.2	1.518	1.079	1.407

SHELLS FROM THE TWO SIDES OF THE ATLANTIC OCEAN

When shells from Bergen, the Cattewater, Church Reef, and Westerly are compared, they are found to be so similar that it is impossible to separate them.

In average size of individuals the four lots vary considerably, those from the Cattewater being the smallest and those from Church Reef, only six miles away, the largest. Among the Cattewater shells, however, there were nine very large individuals illustrated by No. 6 in Fig. 6. These presumably represent a previous generation or age-group, and they have not been included in the Cattewater shells in Tables II and III and in Figs. 4 and 5. Table III and Fig. 5,

giving the numbers of shells at the different proportions, show the amount of overlap between the four lots. Bergen and Cattewater shells are very similar, and the curves for Church Reef and Westerly are well-nigh identical. The Cattewater lot are somewhat more globular than the others, but they are probably younger, if the giant shells like No. 6 are really of the mature adult size. These nine large Cattewater shells have apparently lived an exceptionally long time, for they are the largest *Littorina obtusata* in the extensive collection at Harvard, with the exception of an individual specimen from Cornwall.

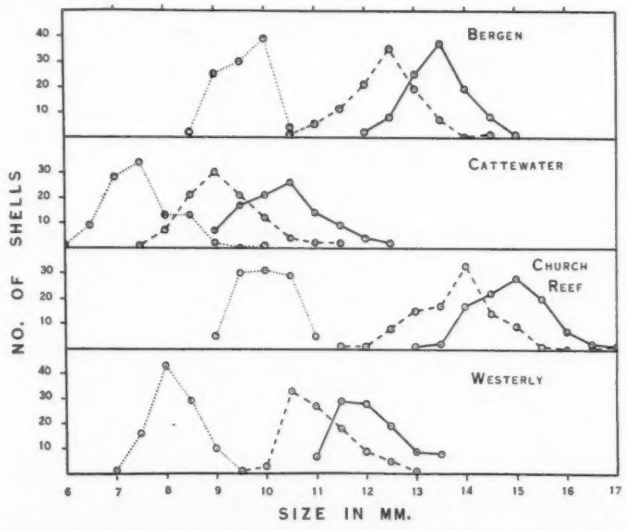

FIG. 4. Shells from the two sides of the Atlantic: numbers of shells of different sizes (See Table II). — = *a*, ··· = *b*, --- = *c*.

In Fig. 6 are shown camera lucida drawings of the obverse and reverse aspects of three shells from each locality, the relative height of the spire to the rest of the shell increasing from left to right. The dimensions and proportions of the same twelve shells are given in Table IV, where the ratio $\frac{a}{c}$ decreases in each group with the increase of the spire component, *c*.

From these figures and tables it will be seen that the amount of variation is much the same in each locality and that it would be quite impossible to sort a mixture of shells from the four places, either on

sight or after measurement. There is then no doubt of the conspecificity of *Littorina obtusata* from Bergen, from the Plymouth district and from Rhode Island.

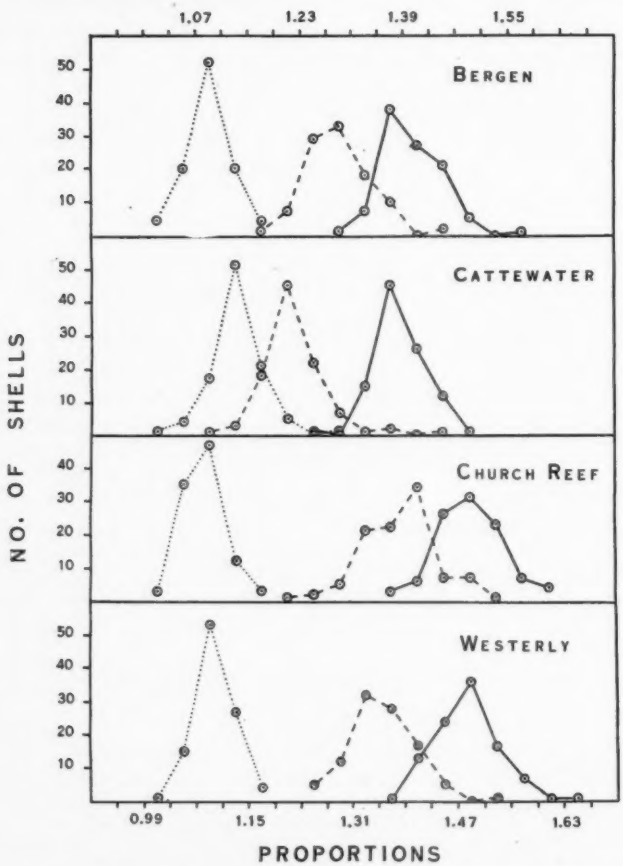

FIG. 5. Shells from the two sides of the Atlantic: numbers of shells of different proportions (See Table III). $— = \frac{a}{b}$, $\cdots = \frac{a}{c}$, $--- = \frac{c}{b}$.

SHELLS FROM THE NEW ENGLAND COAST

It has now been shown that the same species occurs on both sides of the North Atlantic. It remains to be proved that the same species extends up the American Coast, where shells called "*L. palliata*" or

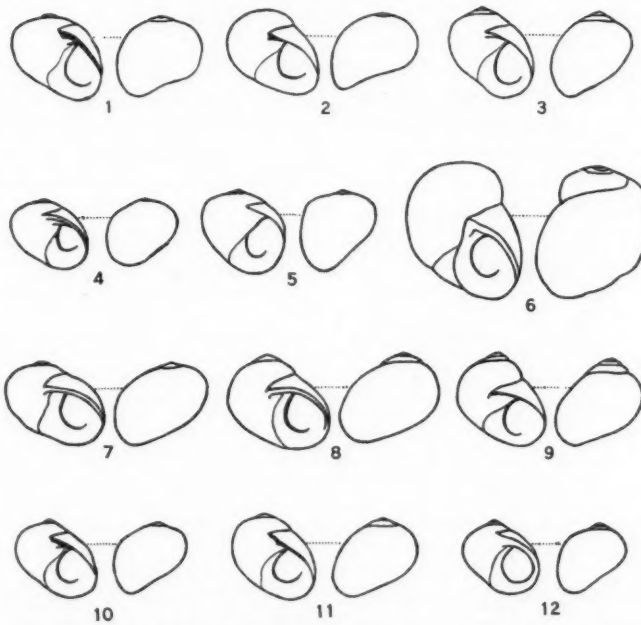

FIG. 6. Shells from the two sides of the Atlantic: obverse and reverse aspects of shells whose dimensions are given in Table IV.

TABLE IV

Shells from the two sides of the Atlantic: dimensions and proportions of shells illustrated in Fig. 6.

Locality	Length (a)	Breadth (b)	Spire to Lip (c)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
	mm.	mm.	mm.			
1. Bergen	14.1	10.5	12.8	1.343	1.102	1.219
2. Bergen	14.1	9.9	13.0	1.424	1.085	1.313
3. Bergen	13.8	9.7	13.0	1.422	1.062	1.340
4. Cattewater	11.5	8.3	9.9	1.386	1.161	1.192
5. Cattewater	12.5	9.3	11.7	1.344	1.069	1.258
6. Cattewater	19.1	13.0	18.9	1.469	1.010	1.454
7. Church Reef	14.9	9.5	12.9	1.569	1.155	1.358
8. Church Reef	15.5	10.5	14.6	1.476	1.061	1.391
9. Church Reef	13.7	9.1	13.5	1.505	1.015	1.484
10. Westerly	12.4	8.3	11.3	1.494	1.097	1.361
11. Westerly	13.2	9.0	12.3	1.467	1.074	1.367
12. Westerly	11.2	7.9	10.5	1.418	1.067	1.329

"*L. obtusata palliata*" occur from Long Island Sound to Newfoundland.

Between shells from Westerly, R. I., and Isle au Haut, Me., there is a considerable difference, namely that between the varieties *littoralis* and *palliata* as described by Dautzenburg and Fischer (1915). These are illustrated in Fig. 7, which represents shells 10 and 33 in Table VII and Fig. 10. Without intermediate forms these might well be considered separate species, the most obvious differences being the thinner shells and the taller spires of the Maine forms. If shells from intermediate places are interpolated, however, a gradual series

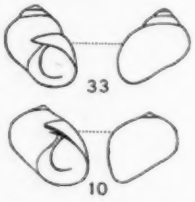

FIG. 7. Shells 10 and 33 from Fig. 10, to show the approximate limits of variation in New England shells.

is obtained between the two extremes, which makes any subdivision impossible. This is clearly shown in Fig. 9, in which the modes of the curves gradually pass from the arrangement in the Westerly lot to that from Isle au Haut, with no sudden transition anywhere. The numbers from which Figs. 8 and 9 are compiled are given in Tables V and VI respectively. In Fig. 10 are shown the obverse and reverse aspects of 24 shells, three from each New England locality, arranged as in Fig. 6 in ascending height of spire from left to right. The corresponding dimensions and proportions are given in Table VII.

Shells 10 and 11 correspond to *L. obtusata littoralis* (D. and F.), Shells 17, 18, 20 and 21 to *L. obtusata typica* (D. and F.), and Shells 29, 30, 32 and 33 to *L. obtusata palliata* (D. and F.). By Winckworth's (1922) reckoning, Shells 10 and 11 belong to a separate species, *L. littoralis*. These figures show, however, that these shells are all part of a continuous series, and any distinction into species or varieties must be purely arbitrary and taxonomically invalid.

Indeed, such bestowal of specific and varietal names and ranks on forms whose differences have not been analyzed would, if carried to the logical conclusion, necessitate the granting of a separate name to every individual (since no two are alike), with an accompanying increase in nomenclatural complexity.

TABLE V

New England shells: numbers of shells of different sizes. (See Fig. 8.)

Locality	Westerly Rhode Island			Cohasset Mass.			Briar Neck Mass.			Rye Beach N. H.			Broad Cove Maine			Port Clyde Maine			Tenants Hbr. Maine			Isle au Haut Maine		
No. of Shells	100			100			86			75			38			74			60			100		
Millimeters	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
5.25 to 5.75.....					3			1												10			4	
5.75 to 6.25.....					14			1			1									26			32	
6.25 to 6.75.....					38			4			1			2			2			18			50	
6.75 to 7.25.....		1			30			9			5			1			4			5			12	
7.25 to 7.75.....		16			13			16		1	12	1		11			8			1	1		2	
7.75 to 8.25.....		43			2	5		25	2		31			18			20		4		8			
8.25 to 8.75.....		29		8		16	1	20	3		21	2		3			27		9		12			2
8.75 to 9.25.....		10		16		28	2	7	5	1	4	2		3	2		13	5	14		14	6		18
9.25 to 9.75.....		1	1	19		21	4	2	5	2			2			4		1	16		14	30		18
9.75 to 10.25.....			6	25		19	6	1	12	1		5			5	2		3	11		3	37		38
10.25 to 10.75.....			33	20		9	10		16	3		12	11		10	1		5	3		6	20		18
10.75 to 11.25.....	7		27	8		2	7		18	17		21	4		9	6		9	1			6		4
11.25 to 11.75.....	29		18	4			21		5	21		23	16		7	10		14	2		2	1		2
11.75 to 12.25.....	28		9				13		12	15		8	2		3	17		14						
12.25 to 12.75.....	19		5				9		5	9		1				16		11						
12.75 to 13.25.....	9		1				8		2	4			1		1	13		9						
13.25 to 13.75.....	8						3		1	1			2		1	5		2						
13.75 to 14.25.....							3		1									1						

TABLE VI

New England shells: numbers of shells of different proportions. (See Fig. 9.)

Locality	Westerly Rhode Island			Cohasset Mass.			Briar Neck Mass			Rye Beach New Hampshire			Broad Cove Maine			Port Clyde Maine			Tenants Hbr. Maine			Isle au Haut Maine		
Number of Shells	100			100			86			75			38			74			60			100		
Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.87 to 0.91																							1	
0.91 to 0.95											1									1			5	
0.95 to 0.99					5									3			11			10			15	
0.99 to 1.03		1			17			5			20			16			19			18			44	
1.03 to 1.07		15			44			32			34			15			29			25			31	
1.07 to 1.11		53			33			44			18			4			15			6			2	
1.11 to 1.15		27			1			6			2												2	
1.15 to 1.19		4																1						
1.19 to 1.23																								
1.23 to 1.27			5			2			2							1								
1.27 to 1.31			12			9			10	1		8			2			2						
1.31 to 1.35			32	1		17			28			19	1		9			8			1			
1.35 to 1.39	1		28	8		21	5		28	5		24	3		12	4		15			3			2
1.39 to 1.43	13		17	24		27	21		12	24		13	17		7	7		17	2		7	2		7
1.43 to 1.47	24		5	24		11	27		6	32		7	11		5	24		14	5		18	3		11
1.47 to 1.51	36			24		8	29		1	8		3	6		2	28		10	21		14	19		17
1.51 to 1.55	17		1	8		3	4			4		1			1	8		5	20		9	20		12
1.55 to 1.59	7			8		1	1			1						1		1	10		3	20		21
1.59 to 1.63	1			3		1										1		1			2	17		12
1.63 to 1.67	1																		2		1	14		10
1.67 to 1.71																					1	4		4
1.71 to 1.75																					1	1		3
1.75 to 1.79																								1

As a final demonstration of the essential unity of *Littorina obtusata* over this considerable geographic range, embracing, as it does, both southern Norway and New England, the data of all the 933 shells from the eleven localities may be added together. This is done in

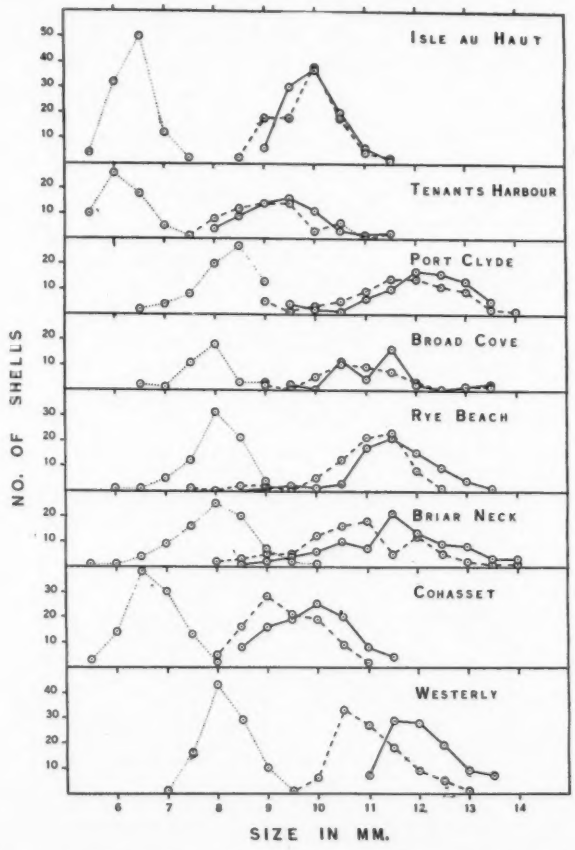

FIG. 8. New England shells: numbers of shells of different sizes (See Table V). — = *a*, ··· = *b*, --- = *c*.

Table VIII, and the result shown in Fig. 11. Although the chief distinction between so-called varieties of *L. obtusata* is usually the variation in the relative height of the spire, the curve $\frac{a}{c}$ in Fig. 11,

giving the ratio of Length to Distance from Spire to Lip, is an almost perfect frequency curve, because the series from blunt to elevated

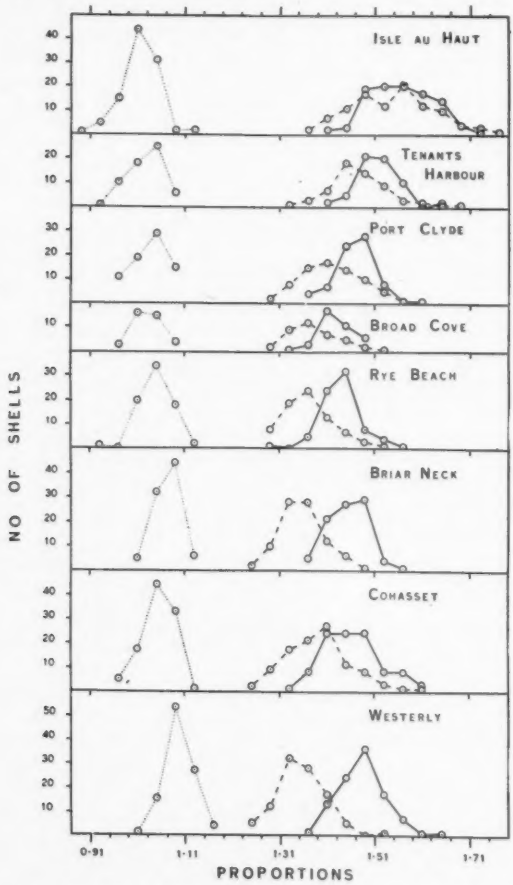

FIG. 9. New England shells: numbers of shells of different proportions (See Table VI). — $= \frac{a}{b}$, ··· $= \frac{a}{c}$, --- $= \frac{c}{b}$.

spires is continuous, with no sudden jumps. The "average" *L. obtusata*, according to these figures, will have the proportions:

$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
1.47	1.07	1.37

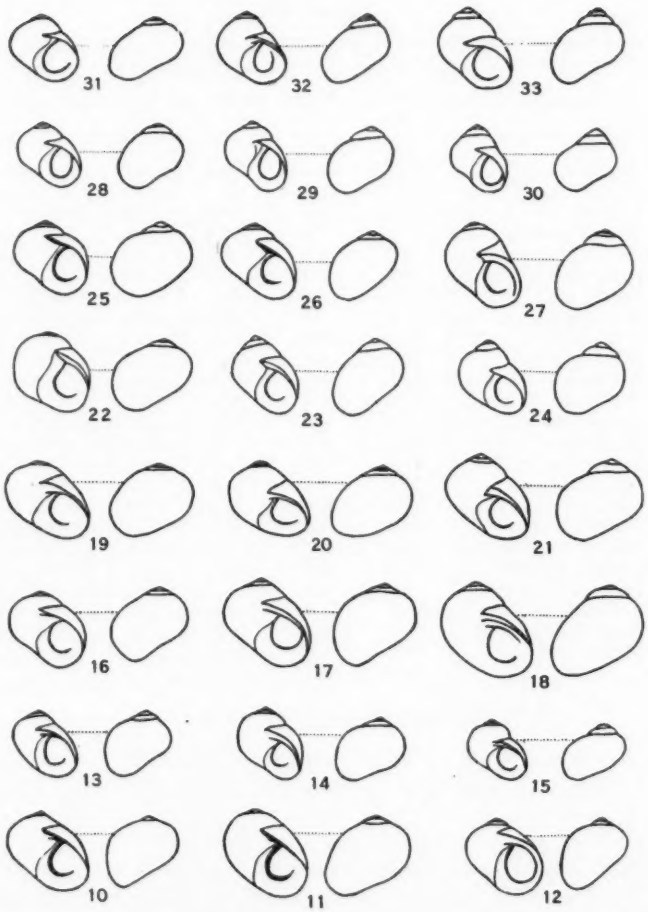

FIG. 10. New England shells: obverse and reverse aspects of shells whose dimensions are given in Table VII.

In conclusion, I wish to thank Mr. W. J. Clench, Curator of Mollusks in Harvard University, for his advice, criticism and kindness.

TABLE VII

New England shells: dimensions and proportions of shells illustrated in Fig. 10.

Locality	Length (a)	Breadth (b)	Spire to Lip (c)	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
	mm.	mm.	mm.			
10. Westerly	12.4	8.3	11.3	1.494	1.097	1.361
11. Westerly	13.2	9.0	12.3	1.467	1.074	1.367
12. Westerly	11.2	7.9	10.5	1.418	1.067	1.329
13. South Cohasset	10.4	7.0	9.8	1.486	1.059	1.400
14. South Cohasset	10.3	7.0	9.9	1.471	1.040	1.414
15. South Cohasset	9.3	6.0	9.0	1.560	1.033	1.500
16. Briar Neck	11.8	8.0	10.8	1.475	1.092	1.350
17. Briar Neck	13.0	9.0	12.4	1.444	1.048	1.378
18. Briar Neck	14.2	9.4	14.0	1.511	1.014	1.489
19. Rye Beach	12.8	8.7	11.7	1.471	1.094	1.345
20. Rye Beach	11.7	8.1	11.1	1.444	1.064	1.371
21. Rye Beach	13.0	8.5	12.4	1.530	1.048	1.459
22. Broad Cove	12.9	8.9	11.9	1.450	1.084	1.337
23. Broad Cove	11.7	8.0	11.7	1.462	1.000	1.462
24. Broad Cove	11.5	7.7	11.9	1.494	0.966	1.545
25. Port Clyde	12.4	8.5	11.5	1.459	1.078	1.353
26. Port Clyde	11.9	7.9	11.2	1.506	1.063	1.418
27. Port Clyde	12.4	8.7	12.6	1.425	0.984	1.448
28. Tenants Harbour	10.0	7.0	9.7	1.429	1.031	1.386
29. Tenants Harbour	10.2	6.9	10.2	1.479	1.000	1.479
30. Tenants Harbour	9.3	6.2	9.7	1.500	0.959	1.564
31. Isle au Haut	10.5	6.8	9.8	1.544	1.072	1.441
32. Isle au Haut	10.5	6.8	10.3	1.544	1.020	1.515
33. Isle au Haut	11.5	7.1	11.7	1.619	0.983	1.647

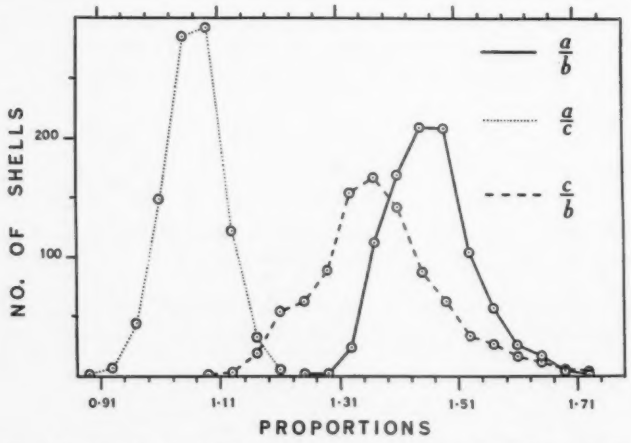

FIG. 11. Nine hundred and thirty-three shells from eleven localities on both sides of the Atlantic: numbers of shells of different proportions (See Table VIII).

TABLE VIII

Nine hundred and thirty-three shells from eleven localities on both sides of the Atlantic: numbers of shells of different proportions.

Proportions	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{c}{b}$
0.87 to 0.91		1	
0.91 to 0.95		7	
0.95 to 0.99		44	
0.99 to 1.03		148	
1.03 to 1.07		284	
1.07 to 1.11		291	1
1.11 to 1.15		121	3
1.15 to 1.19		32	19
1.19 to 1.23		5	53
1.23 to 1.27	2		62
1.27 to 1.31	2	1	88
1.31 to 1.35	24		154
1.35 to 1.39	112		167
1.39 to 1.43	169		141
1.43 to 1.47	209		87
1.47 to 1.51	208		62
1.51 to 1.55	104		33
1.55 to 1.59	56		26
1.59 to 1.63	26		16
1.63 to 1.67	17		11
1.67 to 1.71	4		5
1.71 to 1.75	1		4
1.75 to 1.79			1

SUMMARY

1. *Littorina obtusata* from Norway, from the Plymouth district in England, and from Rhode Island are so alike that they cannot be separated.

2. *L. obtusata* from Rhode Island are fairly unlike those from Maine, but the examination of forms from intermediate localities establishes a continuous series up the New England coast. The range of variation remains roughly constant.

3. Further confirmation of the unity of *L. obtusata* from this wide geographical range is found by adding together the data from all the 933 shells examined. Their proportions follow almost perfect monomodal frequency curves.

4. The names *L. littoralis* (L.) and *L. palliata* (Say) must therefore go into synonymy under *L. obtusata* (L.), since it is shown that there is no division possible between forms to which these names have been given. The name *L. rudis* (Maton) must be put into synonymy,

under *L. saxatilis* (Olivi), as shown by Dautzenburg and Fischer (1912). The first definition of the genus *Littorina* is given by Férussac (1822) on p. xi of his Tabl. Syst. gén de l'emb. des moll.

BIBLIOGRAPHY

ADANSON, MICHEL, 1757. Histoire Naturelle du Sénégal. Part 2, p. 167, pl. 12. Paris.

BINNEY, WILLIAM GEORGE. See GOULD, 1870.

DAUTZENBURG, PH., ET H. FISCHER, 1912. Mollusques provenant des campagnes de l'Hirondelle et de la Princesse Alice dans les Mers du Nord. *Res. Camp. Sci. Monaco*, **37**: 187–201.

DAUTZENBURG, PH., ET H. FISCHER, 1915. Étude sur le Littorina obtusata et ses variations. *Jour. de Conchyol.*, **62**: 87–128.

DONOVAN, EDWARD, 1804. The Natural History of British Shells, Including Figures and Descriptions of all the Species Hitherto Discovered in Great Britain, Systematically Arranged in the Linnæan Manner, with Scientific and General Observations of Each. 5 Vols. London. 1800–1804. Vol. I (published last).

FERUSSAC, J. B. L. D'AUDEBARD DE, 1822. Tableaux Systématiques Généraux de l'Embranchement des Mollusques. Paris and London.

FORBES, E., AND S. HANLEY, 1853. A History of British Mollusca and their Shells. 4 Vols. London. Vol. III.

GOULD, AUGUSTUS ADDISON, 1870. Report on the Invertebrata of Massachusetts. 2nd Edition, comprising the Mollusca; edited by William George Binney. Boston, Mass.

JEFFREYS, J. GWYNN, 1865. British Conchology, or an Account of the Mollusca which now inhabit the British Isles and the Surrounding Seas. 5 Vols. London. Vol. III.

JOHNSON, CHARLES W., 1915. Fauna of New England. 13, List of the Mollusca. Occ. Pap. Boston Soc. Nat. Hist.

KINSEY, ALFRED C., 1930. The Gall Wasp Genus Cynips. *Indiana University Studies*, **16**: Nos. 84, 85, 86.

KUESTER, H. C., 1856. Die Gattung Litorina. In Martini und Chemnitz, "Systematisches Conchylien Cabinet," Vol. 2, No. 9.

LAMARCK, J. B. B. A. DE, 1843. Histoire Naturelle des Animaux sans Vertèbres. Vol. IX. 2nd Edition, by G. P. Deshayes and H. Milne-Edwards. Paris and London.

LINNÆUS, CAROLUS, 1758. Systema Naturæ. 10th Edition. Stockholm. Vol. I.

LINNÆUS, CAROLUS, 1788. Systema Naturæ. 13th Edition, by J. F. Gmelin. Lipsiæ.

MARINE BIOLOGICAL ASSOCIATION, 1931. Plymouth Marine Fauna. Plymouth, England.

MATON, WILLIAM GEORGE, 1797. Observations relative chiefly to the Natural History, Picturesque Scenery and Antiquities of the Western Counties of England, made in the years 1794 and 1796. 2 Vols; J. Eaton, Salisbury, England. Vol. I, p. 277.

MATON, WILLIAM GEORGE, AND THOMAS RACKETT, 1804. An Historical Account of Testaceological Writers. *Trans. Linn. Soc. London*, **7**: 119.

MATON, WILLIAM GEORGE, AND THOMAS RACKETT, 1807. A Descriptive Catalogue of the British Testacea. *Trans. Linn. Soc. London*, **8**: 17.

MENKE, CARL THEODOR, 1828. Synopsis Methodica Molluscorum. Pyrmont.

MENKE, CARL THEODOR, 1830. Synopsis Methodica Molluscorum. 2nd Edition.

MONTAGU, GEORGE, 1803. Testacea Britannica, or Natural History of British Shells, Marine, Land and Freshwater; including the most minute: systematically arranged and Embellished with Figures. London.

OLIVI, ABATE GIUSEPPE, 1792. Zoologica Adriatica. Bassano.

SAY, THOMAS, 1822. An Account of some of the Marine Shells of the United States. *Jour. Acad. Nat. Sci. Phila.*, **2**: 221–248.

SCHMIDT, JOHANNES, 1931. Eels and Conger Eels of the North Atlantic. *Nature*, **128**: 602.

SHERBORN, CHARLES DAVIES, 1927. Index Animalium. Vol. 2, 1801–1850. London.

WINCKWORTH, R., 1922. Nomenclature of British Littorinidæ. *Proc. Mala. Soc. London*, **15**: 95.

INHERITED VARIATION ARISING DURING VEGETATIVE REPRODUCTION IN PARAMECIUM AURELIA[1]

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INTRODUCTION

Jennings (1908), and Jennings and Hargitt (1910) have shown that populations of *Paramecium* which are produced from a single individual are uniform in size, form and fission rate. Other investigators have since confirmed these findings in other genera of Protozoa. Increased variation and the production of different stocks have been found to be produced by conjugation by Jennings (1913), Raffel (1930), Jennings, Raffel, Lynch, and Sonneborn (1932). Other investigators, Middleton (1918), Mast (1917), and Jollos (1921), have found inherited variation arising during vegetative reproduction which they attributed to the action of environmental agents. However, most investigators agree that when a clone of *Paramecium* is cultivated under uniform conditions it is usually decidedly uniform. An excellent review of the literature has recently been given by Jennings (1929).

[1] The present contribution is one of a series of studies on the genetics of conjugation and reproduction in Protozoa, in progress by H. S. Jennings, his associates, and students. The author wishes to record his indebtedness to Professor Jennings for his interest and advice throughout the course of the work.

[2] Fellow of the National Research Council.

This paper presents the results of a study of a clone which sporadically produced branches, or lines, which differed markedly from the original type. These lines, which will be described in detail later, differed in many respects from the unaltered lines and these differences persisted for long periods under identical conditions. None of the altered lines was ever observed to revert to the original type although some were cultivated for nearly 100 generations.

Materials and Methods

The organisms used in this investigation were members of a clone of *Paramecium aurelia* descended by fission from a single ex-conjugant (known as 128*a*) obtained July 15, 1930, in the course of another investigation.

The methods of cultivation employed were the same as those used in the author's (Raffel, 1930) earlier work except that it was found necessary to add a small quantity of the bacteria to the fresh culture fluid each day. The culture medium consisted of a fluid containing KNO_3, 0.5 gram; K_2HPO_4, 0.06 gram; $MgSO_4.7H_2O$, 0.02 gram; $FeCl_3$, 0.001 gram; H_2O, 1 liter. To this solution cultures of an alga, *Stichococcus bacillaris,* and a bacterium, usually *Achromobacter candicans,* were added. The paramecia were cultivated in this suspension under bacteriological conditions.

Description of the Altered and Unaltered Lines of the Clone

1. *Origin.*

The clone 128*a* differed markedly from the other clones isolated from a group of individuals undergoing conjugation July 15, 1930. It was found to have a characteristic form and size quite diverse from the form and size of the other clones which were studied. It also differed from most of the other clones in its slow rate of reproduction. For these reasons it was selected as one of the few clones to which was devoted further intensive study. Later study has shown that it also differed from most of the other clones in its tendency to give off branches quite different from the main clone and in its ability to withstand adverse environmental conditions.

On August 4, 1930, twenty-two days after this clone was isolated, two of the twenty-four lines which were being cultivated became radically altered in ways to be described below. Three other lines became altered in a similar manner during the next three days. These five lines were discarded on August 15. During the five days following August 29, seven more lines became altered in a similar manner. These were

cultivated until September 18, when they were discarded. No other altered lines appeared until early in November, but from that time until June of the following year, they were produced very frequently.

2. Comparison of the unaltered and altered lines.

The typical individuals of this clone, hereafter referred to as the unaltered lines, differed from the altered lines in the following ways:

(a) Size. One of the most striking differences between the two groups was the difference in size. This difference is well illustrated by Fig. 1, which shows typical specimens of each group under identical

FIG. 1. Camera lucida drawings of typical individuals of (*a*) unaltered and (*b*) altered lines.

conditions. As can be seen from this figure the individuals of the altered lines were more than twice as long as those of the unaltered lines. This marked size difference was a matter of continuous daily observation.

(b) Form. The two branches of clone 128*a* differed markedly in form. The unaltered individuals of this clone were thick in the central region and tapered to points at each end. They were quite pale and usually had a dark food vacuole in the anterior tip. The altered individuals were, on the other hand, of the typical *P. aurelia* form, *i.e.*, somewhat pointed anteriorly, rounded posteriorly with their greatest breadth about two-thirds from the anterior end. Usually they had many food vacuoles distributed chiefly in the posterior part of the organism. These differences in form as well as the size differences are shown clearly in Fig. 1. This figure shows typical adult individuals of

both kinds living under identical conditions and drawn at the same time. The differences in form and also the differences in size persisted even during the periods of depression when the fission rates of the two groups approached one another. At these times the two groups could be readily distinguished by their appearance.

(c) Fission Rate. The altered lines differed from the unaltered lines greatly in their rates of reproduction. The clone originally was a very slowly dividing one with a mean fission rate much below one divi-

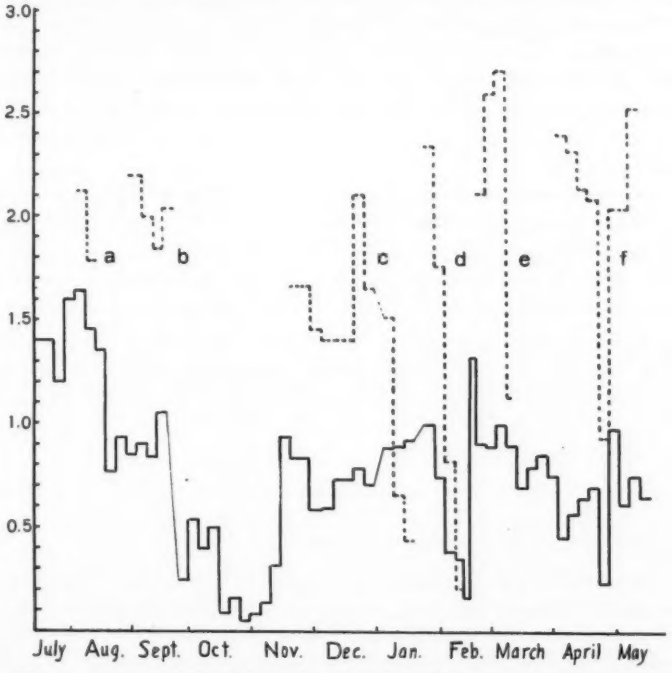

FIG. 2. Average daily fissions of unaltered (solid line) and of altered (dotted lines) lines plotted in periods of approximately five days each.

sion per day. The altered lines, on the other hand, divided very vigorously and some of them averaged well over two fissions per day for periods exceeding two weeks. This difference in fission rates is illustrated in Fig. 2. The solid line in Fig. 2 shows the mean daily fission rates for the original clone from July 15, 1930 to May 17, 1931, plotted in approximately five-day periods. The dotted lines represent the average daily fission rates of six different groups of altered lines which

were studied. These are also plotted for the most part in five-day periods. From this figure it is evident that the altered lines differ markedly from the unaltered lines in their rates of reproduction.

(d) Resistance. The altered lines of clone 128*a* were much less resistant to unfavorable conditions than were the unaltered lines. This can be readily observed from Fig. 2 which shows that in nearly every period of depression the altered lines suffered a decrease in fission rate earlier than the unaltered lines, and that the extent of the depression was greater in the altered lines. For example, group (*c*) Fig. 2 declined in fission rate from 1.26 to 0.43 fissions per day between January 10 and January 20, while the unaltered lines showed an actual increase from 0.88 to 0.93 fissions per day during the same period. Similar though not so great differences can be found by comparing groups (*d*), (*e*) and (*f*) with the unaltered lines which were cultivated simultaneously with them.

We have seen then that the clone 128*a* produced branches during vegetative reproduction which differ markedly in size, form, fission rate, and resistance from the unaltered lines. The differences in size and form persist even during periods of depression during which the fission rates of the two groups were similar. These differences are as great as any found by Jennings, Raffel, Lynch, and Sonneborn (1932) in their extensive study of diversities between biotypes produced by conjugation. The altered lines persisted for long periods and none has ever been observed to revert to the original condition although many thousands of individuals have been examined.

Experiments

1. *Regularity in the production of altered lines.*

The remaining parts of this paper deal with experiments designed to discover whether any regularity was apparent in the production of these altered lines, and whether after conjugation these unlike branches of the same clone would produce similar or dissimilar populations. In order to learn more about the occurrence of the altered individuals and to ascertain whether there is any regularity in their production, the following experiments were performed:

From November 3rd to 19th the progeny of one individual of the clone was expanded to form 336 lines of descent whose relationships were known. During this period some of the lines became altered. These, because of their faster rate of reproduction, gave rise to the greater part of this population. After the desired number had been obtained, the organisms were cultivated under identical conditions for

ten days. During this period the lines which were altered at the beginning passed through from 1–21 generations (all but one of the 226 such lines which lived until November 29 passed through from 11–21 generations), while the unaltered lines passed through from 4–9 generations. As is shown in Fig. 2 (*c*), the mean daily fission rate for the altered lines is 1.67 as compared with 0.83 fissions per day for the unaltered lines.

On November 29 all except one of each of the altered and unaltered lines were discarded. Then each of these was expanded to a population of 168 individuals. These two populations were then cultivated for ten days from December 11–21, during which time they showed great diversity in their size, forms and fission rates (*see* Table I). Although

TABLE I

Distribution of total fissions December 12–21, 1930, of populations derived from (a) a single individual which manifested the altered set of characteristics on November 18, and (b) from an individual of the original type.

Fissions	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Mean
Altered lines	1			1		1	3	3	7	7	2	3	9	12	12	24	13	10	4	13.1
Unaltered lines	0	1	5	10	12	17	18	8	6	5	5	5	6	5	4	3	2			7.2

the altered lines were much depressed at this time because of unfavorable environmental conditions or endomixis, the population derived from the altered individual produced no individuals which were like the unaltered lines.

Figure 3 is a genealogical chart showing the relationships of the lines descended from the unaltered individual, with their numbers of fissions during the ten days following December 11. These lines were all of the original type on December 11; but, as is shown, many of them, represented by the filled-in circles on Fig. 3, became altered subsequently.

From the data given in Fig. 3, an attempt was made to ascertain whether or not there is any regularity or rule in the production of altered stocks. That is, if a given line is altered, are the lines which are separated from it by fewest fissions more likely to be altered than lines separated by more fissions or not; or is any other rule to be found which would describe the production of the altered lines? To investigate this question the coefficients of correlation were obtained for the total number of fissions between December 12 and December 21, of lines represented on Fig. 3 which were separated on December 11 by one, two and three fissions. The lines which were separated by but one fission at this time showed a correlation of 0.56 ± 0.06; those separated by two fissions a correlation of 0.23 ± 0.12; while those separated by three fis-

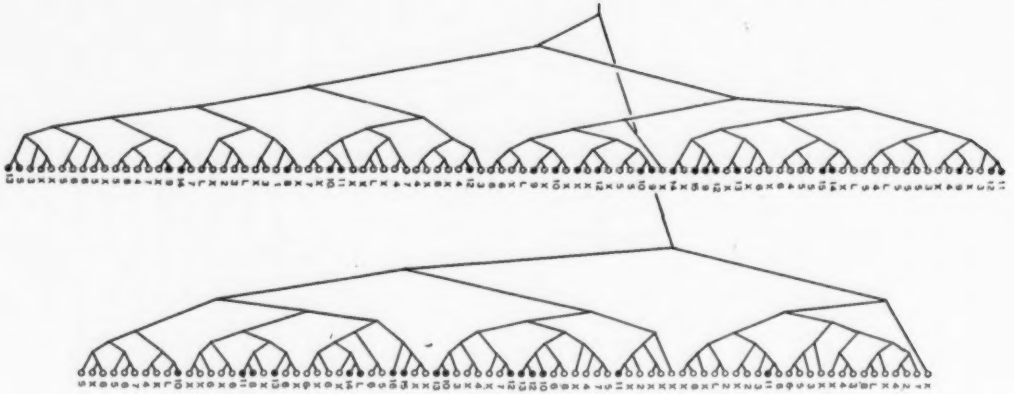

FIG. 3. Genealogical chart showing the relationships of the lines derived from an unaltered individual between November 29 and December 11, 1930, with the number of fissions of each line from December 12–21 inclusive. The lines were all unaltered on December 11. Those which remained unaltered are represented by open circles, those which became altered by solid circles.

X indicates that the line died before December 21.

L indicates that the line was lost.

sions gave a coefficient of correlation of 0.48 ± 0.06. Thus there is apparently no relation between the number of fissions by which lines are separated and the fate of the lines. The decrease in the coefficients of correlation of the lines separated by two fissions as compared to those which are separated by only one appears to be meaningless when we find that those separated by three fissions do not differ significantly from those separated by only one fission. A careful study of the records, however, shows that when two lines are derived from a common ancestor, one or two generations before one of them becomes altered the other usually becomes altered in a similar manner at nearly the same time. This occurred frequently throughout the entire six months that this subject has been intensively investigated.

The experiments and observations which were made with the object of discovering any regularity or rule in the production of altered lines did not give any evidence of any such regularity. They did show, however, that if a line becomes altered, lines which were derived from the same individual not more than two generations previously generally became altered at the same time or at nearly the same time.

2. *Effect of conjugation in the diverse lines.*

In another paper Jennings, Raffel, Lynch, and Sonneborn have shown that when two diverse clones conjugated they produced two very diverse populations. In the two clones intensively studied, although each population showed great variation in fission rates, the mean fission rate of each was not very different from the fission rate of the clone which produced it. . In the particular cases which they studied the mean fission rates of the two populations differed in the same direction and to approximately the same extent from the mean fission rates of the clones from which they came. In the present investigation we have two races which differ more than the two clones studied by Jennings, Raffel, Lynch, and Sonneborn. These two races are, however, not different clones but are branches of the same clone; that is, they are descended by vegetative reproduction from a common ancestor. Will these two very diverse races also produce by conjugation populations which are on the whole diverse, or will they, unlike diverse clones, produce populations which are similar? In order to answer this question typical lines of the two races of this clone were chosen and expanded in isolation drop cultures until large numbers of both types were available. Then conjugation was induced in both branches of the clone and 105 pairs of conjugants and 96 non-conjugants were taken in each group. In the case of the slowly reproducing unaltered lines care was taken to choose among the non-conjugants some split pairs and others which were be-

ginning to conjugate. These four sets of organisms were then cultivated side by side for ten days after the last ex-conjugants were isolated.

The results of this experiment are shown in Figs. 4 and 5. Figure 4 shows the course of the mean daily fission rate of the two groups of non-conjugants; and Fig. 5 shows the same for the ex-conjugants. As can be seen, the non-conjugants of the unaltered lines continued to reproduce at the rate of approximately 0.9 fissions per day, while the ex-conjugants from this group reproduced much more rapidly, reaching at the end of the experiment a rate of 1.6 fissions per day. This progressive increase in fission rate was due entirely to the dying of the many abnormal lines which were produced by conjugation. Ninety-three non-viable lines were produced. Only 10 of 210, or 4.8 per cent, of the ex-conjugant lines resembled the parent race in fission rate.

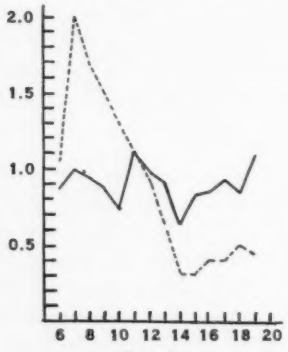

FIG. 4

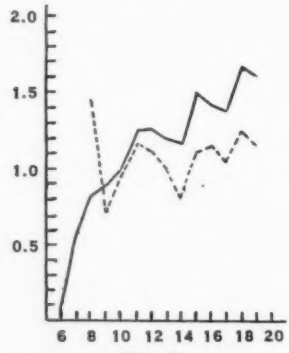

FIG. 5

FIG. 4. Average fissions on successive days of non-conjugant lines of the unaltered (solid line) and altered (dotted line) groups.

FIG. 5. Average fissions on successive days of the ex-conjugant lines of the unaltered (solid line) and the altered (dotted line) groups.

Among the 96 lines of the non-conjugants, 29 became altered and 24 lines died out during this period.

The results obtained from the altered lines were quite different. The non-conjugants at the beginning were very healthy and vigorous, reproducing at the rate of two fissions per day the second day after conjugation and nearly as rapidly the next day. From then on they declined rapidly in vigor—the fission rate falling to between 0.3 and 0.5 fission per day, accompanied by a great increase in mortality. The ex-conjugants, which were also very vigorous at first, with a fission rate of 1.4 for the first day, declined to approximately one fission per day

in spite of the death of the 90 non-viable lines which had been produced by conjugation.

Because of the depressed conditions of the organisms during this experiment it seemed advisable to repeat it under more favorable conditions. Great difficulty was experienced in trying to obtain the conjugants from the unaltered lines as these were constantly producing organisms of the altered type, which in a short time would greatly outnumber the type which the culture originally contained. Finally, in order to know which type of organisms was conjugating in the cultures of the original group, split pairs were taken at the same time as the conjugants. Thirty-three per cent of all of the organisms obtained from split pairs were of the original type. However, no pair among the split pairs was composed of two organisms of this type. It is very probable that this was due in a great measure to the subsequent altera-

TABLE II

Distribution of fissions during the first five days after conjugation of ex-conjugants derived from (a) altered lines and (b) unaltered lines of clone 128*a*.

Fissions	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	No. of lines	Mean
Altered lines	4	0	1	1	2	3	3	9	10	14	17	10	24	33	19	1	151	10.5
Unaltered lines	0	4	2	3	4	11	6	6	8	8	19	18	38	46	24	19	216	10.9

tion of one member of the pairs, as altered lines were being produced rapidly at this time.

The results of the conjugations in the two branches were as follows: The unaltered lines gave rise to a population very different from themselves. The mean fission rate of 2.2 fissions per day is much greater than the 0.62 fission per day of the non-conjugants which lived through the same period. The ex-conjugants were somewhat less viable than the non-conjugants of this group; 22.3 per cent of the former died during this period as compared to 18.9 per cent of the non-conjugants. Only 11 of the 278 ex-conjugant lines or 4 per cent had the shape and size of the unaltered lines.

On the other hand, the altered lines gave a quite different result. The ex-conjugant lines were much less viable than the non-conjugants; 34.7 per cent of the lines derived from ex-conjugants died, while there was no mortality in the non-conjugants. The mean fission rate of the ex-conjugants was 2.1 fissions per day as compared with 2.3 for the non-conjugants. No single individual among the ex-conjugant lines obtained from the altered group had the size or form of the unaltered lines. A comparison of the two groups of ex-conjugants with respect to their fission rates is given in Table II. This table shows the great

similarity between the fission rates of the two populations which have the same mode and means which differ very slightly.

These results differ markedly from those obtained by Jennings, Raffel, Lynch, and Sonneborn, on the effects of conjugation in their two diverse clones. The two populations produced after conjugation by the two diverse branches of the same clone, in the investigation presented here, are nearly identical with respect to their fission rates. The mean fission rates of both populations are nearly equal to the mean fission rate of the altered population. In the case of the altered lines this result is in accordance with the results obtained by earlier workers. Jennings (1913) and Raffel (1930) found that conjugation within a clone results in a population showing much variation, with a mean fission rate slightly lower than that of the parent clone, and an increase in mortality. In the case of the unaltered lines of this clone the effect of conjugation is to produce a population quite different from the non-conjugant population. The mean fission rate is increased to more than three times the mean fission rate of the non-conjugants and the size and shape of 96 per cent of the ex-conjugants is altered. Thus the unaltered lines of clone 128*a* are affected by conjugation in a way quite unlike most of the clones which have previously been described.

It is obvious that the two very diverse branches of clone 128*a* are affected quite differently by conjugation. Instead of giving rise to two diverse populations as did the two diverse clones investigated by Jennings, Raffel, Lynch, and Sonneborn, they produced, after conjugation, populations nearly identical in fission rates and appearance. This indicates certainly that the inherited diversity which appeared during vegetative reproduction in this clone differs in some way from the diversities between the clones studied by Jennings, Raffel, Lynch, and Sonneborn.

Discussion and Conclusions

The inherited variations which arose in this clone during vegetative reproduction differ markedly from those which are produced by conjugation. The altered lines which were produced were similar to one another in all respects and they were quite different from the original type, while conjugation produces from a single clone a large number of clones which differ much or slightly from one another so that a more or less continuous series is produced. The differences between the two branches of this clone are to a great degree eliminated by conjugation while diverse clones produced by conjugation usually yield after subsequent conjugations populations which are similar to themselves in their fission rates and other characteristics. This suggests that the basis of

heritable variation found in this clone is different from that of the variation produced by conjugation.

The increase of variation after conjugation and the cytological details of that process have led to the conclusion that conjugation involves biparental inheritance and that the increased variation produced by conjugation is brought about by a recombination of genetic factors. The inherited variation reported in this paper, however, does not seem to arise from such recombinations. It differs from the variations produced in that manner as set forth above; and in order to ascribe its origin to such recombinations it would be necessary to postulate some such process for this particular clone only. Therefore, it seems that recombinations must be dismissed as a possible explanation of the origin of this variation.

That the genetic constitution of the original clone must contain a number of heterozygous pairs of genes is evident from the results of conjugation between members of the unaltered branch. In order that a particular clone should produce, when inbred, a population only 4 per cent of which resembles the parent, it is necessary that the clone should be heterozygous for four or five genes. Therefore, we can assume that a heterozygous condition of four or five pairs of genes is necessary for the production of the unaltered type of this clone. A mutation then of any of eight or ten genes would produce an altered line in this clone. It is possible, however, that the observed altered lines could only be produced by the mutation of any of the four or five "mutant" genes to the "normal" condition (as a mutation of the "normal" gene might be lethal when homozygous). The evidence leads to the conclusion that the production of altered lines in this clone is probably caused by the mutation of one of the members of the four or five pairs of heterozygous genes.

This conclusion explains all of the phenomena observed. There is no regularity in the production of the altered line—no regularity would be expected of gene mutations. The altered lines never produce the original type after conjugation—as it is not heterozygous for all five pairs, it cannot produce progeny which are heterozygous for all five pairs.

The only question which this conclusion raises is the frequency of gene mutation in *Paramecium*. This is to be the subject of a subsequent paper, but it might be said here that this explanation of the questions raised by this investigation throws some light on other questions. Jennings, Raffel, Lynch, and Sonneborn found that clones differ in their uniformity. Others have found variation between clones in mortality, etc. If gene mutations are comparatively frequent in *Paramecium*, it

can account for the cases of inherited variation reported by other workers as well as the unexplained mortality that occurs in all isolation culture work. It, furthermore, can explain the differences in mortality which are observed in different clones. The only alternative to this explanation is that the original clone contained a detachable translocation which was lost in the production of the altered branches. The former explanation seems preferable because it explains the numerical relationships found in this investigation as well as the other phenomena which have been mentioned; and also because much evidence has subsequently been obtained which indicates frequent mutations in *Paramecium*. A more detailed treatment of these questions will be presented in the near future.

Summary

The clone of *Paramecium aurelia* studied in this investigation produces branches which differ from the original clone in many respects. They are larger, have a different form, reproduce at a greater rate, and are less resistant to unfavorable conditions than the original type. These branches consistently manifest their diverse characteristics and none has ever been known to revert to the original type in any of the cases which were studied. No rule or regularity was found in the production of the altered lines except that when a line became altered, lines which were separated from it by only one or two fissions usually became altered at the same time or nearly the same time. Unlike diverse clones, these diverse races of the same clone give after conjugation populations which are on the whole very similar. The unaltered type gave after conjugation only a very small proportion (4 per cent) of lines which were similar in size, form and fission rate to the parent clone.

From these observations and experimental results the conclusion is drawn that the variation is produced by mutations of one member of the four or five heterozygous pairs of genes which are necessary to produce the normal type of this clone.

LITERATURE CITED

Jennings, H. S., 1908. Heredity, Variation and Evolution in Protozoa. II. Heredity and variation of size and form in Paramecium, with studies of growth, environmental action, and selection. *Proc. Am. Philos. Soc.*, **47**: 393.

Jennings, H. S., 1913. The Effect of Conjugation in Paramecium. *Jour. Exper. Zool.*, **14**: 279.

Jennings, H. S., 1929. Genetics of the Protozoa. *Bibliographia Genetica*, **5**: 105.

Jennings, H. S., and G. T. Hargitt, 1910. Characteristics of the Diverse Races of Paramecium. *Jour. Morph.*, **21**: 495.

JENNINGS, H. S., D. RAFFEL, R. S. LYNCH, AND T. M. SONNEBORN, 1932. The Diverse Biotypes Produced by Conjugation within a Clone of Paramecium aurelia. (In press.)

JOLLOS, V., 1921. Experimentelle Protistenstudien. I. Untersuchungen Uber Variabilität und Vererbung bei Infusorien. *Arch. f. Protist.*, **43**: 1.

MAST, S. O., 1917. Mutation in Didinium nasutum. *Am. Nat.*, **51**: 351.

MIDDLETON, A. R., 1918. Heritable Effects of Temperature Differences on the Fission Rate of Stylonychia pustulata. *Genetics*, **3**: 534.

RAFFEL, D., 1930. The Effect of Conjugation within a Clone of Paramecium aurelia. *Biol. Bull.*, **58**: 293.

RACIAL DIFFERENCES IN THE EARLY PHYSIOLOGICAL EFFECTS OF CONJUGATION IN PARAMECIUM AURELIA

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I. INTRODUCTION

The Problems and Their Present Status

The question of the effect of conjugation has usually been proposed in the form: "What is the effect of conjugation in the ciliate Protozoa?" The possibility that different species might show different effects of conjugation has been suggested, but the possibility that similar differences might be found between different lines of descent within a single species has received but scant attention. Over forty years ago Maupas (1888) attempted to demonstrate that conjugation between unrelated (or distantly related) individuals was fertile, but that conjugation between close relatives was not. Calkins (1919 and 1920), working on *Uroleptus mobilis,* and Woodruff and Spencer (1924), working on *Spathidium spathula,* found that conjugation in much depressed lines did not usually bring the ex-conjugants to so high a reproductive rate

[1] The present contribution is one of a series of studies on the genetics of conjugation and reproduction in Protozoa, in progress by H. S. Jennings, his associates, and students. The authors wish to record their indebtedness to Professor Jennings for his interest and advice throughout the course of the work. This investigation was aided by a grant from the Bache Fund.

as did conjugation in the less depressed lines. In other words, there was a positive correlation between the mean fission rates of the non-conjugants and the mean fission rates of the derived ex-conjugants. MacDougall (1931) has shown that morphologically different races of *Chilodon uncinatus* yield similar morphologically different groups of ex-conjugants. Jennings *et al.* (1932) have shown that in *Paramecium aurelia* two ex-conjugant clones which differ markedly in mean fission rate yield groups of ex-conjugants which differ similarly in mean fission rate. Raffel (1932), on the other hand, working on a single ex-conjugant clone of the same stock of *Paramecium aurelia* employed by Jennings *et al.*, found that two sub-clones with considerably diverse fission rates which arose from this single clone during vegetative reproduction, yielded groups of ex-conjugants that were not significantly different. (It needs scarcely to be recalled that in the work of Jennings *et al.*, and of Raffel, great differences were found within each group of ex-conjugants; the values compared are merely mean values. See the original papers for details.)

These results of Woodruff and Spencer, MacDougall, and Jennings *et al.* strongly support the idea that the effects of conjugation may differ in different lines of descent within the same species. Only in the work of MacDougall, however, has there been repeated demonstration in the same races, of the same differences in the effects of conjugation. In her work, the differences studied were morphological. There is on record no thorough study of characteristic racial differences in the effects of conjugation on fission rate, variability or mortality. These physiological characteristics are the ones most intimately related to current theories of conjugation. Studies on characteristics such as these are required in order to determine whether the differences in results, and in theories constructed from these results, as set forth by different investigators, are due partly or entirely, not to specific differences, but merely to racial differences in the stocks they studied. Might conjugation result in rejuvenescence in some races of a species and not in others? Might conjugation result in great increase in variation in some races of a species and in little or no increase in variation in others? Might conjugation result in great mortality in some races and in little or no mortality in others?

If conjugation in Protozoa is fundamentally similar to zygote formation in higher organisms, then, as suggested by Jennings (1929), conjugation should result in similar recombinations of chromosomes and genes. The present state of knowledge of the genetics of the Protozoa, unlike the state of knowledge of the genetics of higher organisms, does not justify the assumption of genes; but the works of Jennings (1911,

1913), Jennings and Lashley (1913, 1913*a*), Pascher (1916), Raffel (1930), and Jennings *et al.* (1932) demonstrate that conjugation in Protozoa is a process resulting in biparental inheritance and in the formation of many new hereditary types. If the nuclear recombinations in the conjugation of Protozoa further resemble the nuclear phenomena in gamete and zygote formation of higher organisms, racial differences in the effects of conjugation might be predicted. Races which differed in the number of heterozygous "gene" pairs (or whatever the corresponding determiners in Protozoa may be called) would differ in the amount of variation produced by conjugation. Races which differed in the number of heterozygous lethals would differ in the mortality produced by conjugation. Races which differed in the genes determining fission rate would yield conjugants which differed in these respects; thus some races might be rejuvenated by new, favorable, chromosomal recombinations, other races depressed by new, unfavorable chromosomal recombinations.

The importance of the issue for current theories of conjugation, the seeming likelihood of obtaining a result throwing a light on the possible analogy between conjugation in Protozoa and zygote formation in higher organisms, and the fact that encouraging results had already been obtained both in our own work and in the work of others, induced us to study intensively this matter of characteristic physiological differences between races, in the effects of conjugation.

Plan of Investigation

The present investigation begins at the point reached by the results of the investigation of Pascher and of Jennings and his collaborators, mentioned above, and goes on to ask the following questions:

1. When the diverse clones produced by conjugation within a single clone are allowed to conjugate, each within itself, do they all produce similar sets of new ex-conjugant clones, or do the results of conjugation differ in the diverse parent clones? That is, does conjugation produce the same result in slowly multiplying clones as in those that multiply rapidly? Does conjugation produce similar results in clones differing in other respects?

2. If different results are produced, is there a correlation between the characteristics of the parent clones and those of derived ex-conjugant clones? For example, does a slowly multiplying clone produce a set of ex-conjugants that multiply on the average less rapidly than the set produced by a rapidly multiplying clone?

3. Do successive conjugations among the members of a single clone yield similar sets of ex-conjugant clones in each case? Or do different conjugations within the same clone yield diverse results?

4. Does the mean fission rate of the ex-conjugant clones always bear the same relation to the fission rate of the respective parent clone? Or may conjugation in some clones result in an increased fission rate, in other clones in a decreased fission rate?

To answer these questions, the following were studied (compare the diagram of Fig. 1):

A. Comparison of the effects of successive conjugations in the same clone. In the clone employed (designated 247*a*), part of the individuals were allowed to conjugate, while others were not; the two sets were then compared. Later, another set were allowed to conjugate, and these again compared with the non-conjugants. This was repeated for a third time.

B. Comparison of the effects of simultaneous conjugations in six different ex-conjugant clones, which had similar vegetative characteristics.

C. Comparison of the effects of repeated simultaneous conjugations in two of the ex-conjugant clones that had shown diverse results in the first comparison (*B*). In all, four successive simultaneous conjugations of the two stocks were compared.

D. Comparison of the effects of conjugation between the above two clones, with the effects of conjugation within each of these clones. This part of the work is but briefly reported here, as the matter is of such novelty and importance as to warrant treatment in a later separate publication, in which will be given also the results of other crosses that have been made.

In giving the results of these four sets of experiments, it will be observed that the data are mainly for the early effects of conjugation: for those manifested within the first two or three weeks after conjugation. During this early period occur many of the most characteristic effects of conjugation, such as the dying out of weak races. As to the relation between the earlier and later effects of conjugation, the present paper makes no assumptions. A comprehensive study of conjugation requires a knowledge of both its earlier and its later effects. It is planned to have later contributions in this series of investigations deal explicitly with the later results of conjugation, as compared with the earlier ones.

II. Materials and Methods

Throughout the course of the investigation, the paramecia were cultured in isolation, one paramecium to one drop of culture fluid. The culture drops were placed on double-concavity, hollow-ground slides. These slides were kept together in groups of twelve on a glass plate

raised by glass supports above the bottom of a 10-inch inverted Petri dish sealed with distilled water. These chambers of isolation cultures were examined daily, at which time one animal from each culture drop was transferred to a fresh drop of culture fluid on another slide.

At different times during the course of the work three different methods of preparing the culture fluid were employed; these may be referred to as methods *A*, *B*, and *C*. Method *A* is the method described by Jennings *et al.* (1932). This consists of boiling 30 flakes of Quaker Rolled White Oats in 100 cc. spring water for 3 minutes; allowing this to stand five minutes; filtering into 250 cc. Erlenmeyer flask; letting filter and flask stand for 24 hours; then inoculating filtrate (now rich in bacteria from air and glassware) with the alga *Stichococcus bacillaris* (grown on agar slants). Method *B* is the method described by Raffel (1930), except that precautions designed to exclude foreign bacteria were not employed. Raffel's synthetic fluid was inoculated, just before using, with the bacterium *Achromobacter candicans* (grown on agar slants) and the alga *Stichococcus bacillaris* (also grown on agar slants). Culture method *C* is merely a slight modification of method *A*. It differs from method *A* in two particulars: (1) The first part of the filtrate, which comes through the filter rapidly in the first five minutes, is rejected; (2) The 24-hour-old infusion is boiled and cooled just before inoculating with *Stichococcus*. These modifications of method *A* were found necessary at certain times to keep within satisfactory limits the quantity of bacteria in the culture fluid.

The paramecia used in the present study were all descendants of a single ex-conjugant, known as 247*a*, from Raffel's intra-clonal conjugation of July 15, 1931, reported by Jennings *et al.* (1932). All individuals descended from 247*a* by vegetative reproduction without the intervention of conjugation are known as the clone 247*a*. This clone was kept under observation throughout the entire course of the experiments from October 1, 1930, till April 1, 1931. In Fig. 1, which gives a graphic account of the course of the experiments, the continuous base line represents the clone 247*a*. On October 1, October 16, and December 9, parts of the clone 247*a* were allowed to conjugate; this is represented by the oblique lines leading to *C*, *D*, and *E*. The group of conjugants obtained October 1 was called the *C* group; it consisted of 58 pairs. After separation of the members of the pairs each gave rise to a single line of vegetative descent; some of these lines of descent are represented in Fig. 1, by straight lines parallel to the 247*a* line. The *D* group beginning October 16, consisting of 100 lines of descent from 50 pairs of conjugants, and the *E* group beginning December 9, consisting

of 194 lines of descent from 97 pairs of conjugants, are represented in a similar way.

From this *E* group were selected for further examination lines of descent from six of the ex-conjugants. These as shown in Fig. 1, were ex-conjugant clones *E*40*a*, *E*41*a*, *E*46*b*, *E*80*b*, *E*81*a*, and *E*85*b*. On January 12–14, conjugation was simultaneously induced within each of these six ex-conjugant clones. These conjugations, known as *F*, *G*, *L*,

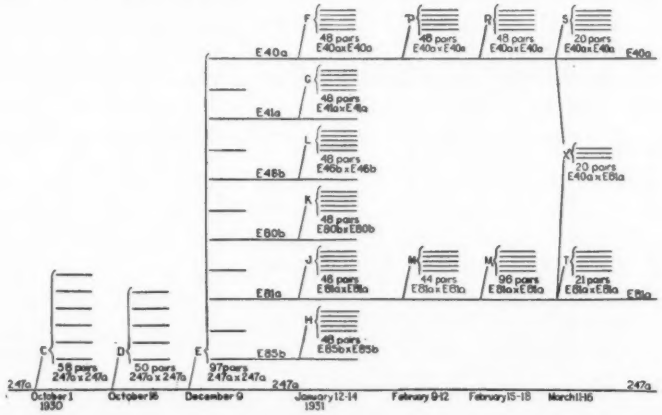

FIG. 1. Pedigree chart of the clones used in this investigation; all clones were derived, as shown, from the clone 247*a*. The horizontal lines represent single ex-conjugant clones (descendants of a single ex-conjugant) multiplying vegetatively without the intervention of conjugation. An oblique line branching off from a line representing a clone indicates that a part of this clone was allowed to conjugate. Each group of conjugants is designated by a single capital letter to which an oblique line leads. The brackets following this letter enclose a set of horizontal lines representing some of the new ex-conjugant clones. Below these lines are given, in parentheses, the number of pairs of conjugants and the clone-names of the conjugating individuals.

K, *J*, and *H*, respectively, each consisted of 48 pairs of conjugants. Two of the six clones, namely *E*40*a* and *E*81*a*, were kept for further comparisons. In these two clones, simultaneous conjugations were induced on February 9–12, February 15–18, and March 11–16. These simultaneous conjugations are designated *P* and *M*, P_1 and M_1, and *S* and *T*, respectively. At the same time as the conjugations *S* and *T* were taking place within the races *E*40*a* and *E*81*a*, conjugation *X* was also taking place. This was a cross-conjugation between representatives of *E*40*a* and *E*81*a*.

In the course of the experiments, sixteen different sets of conjugants and their descendants were studied. These are listed, together with

TABLE I

General Information Concerning the 16 Conjugations Studied

Clones from which conjugant pairs were derived	Name of group of ex-conjugants	Dates of conjugation	Period observed	Number of pairs	No. of lines of each ex-conjugant	Total no. of lines	Temperature range in degrees centigrade	Culture method
247a × *247a*.....	*C*	Oct. 1–2 1930	Oct. 1–14	58	1	116	21°–23°	*B*
247a × *247a*.....	*D*	Oct. 16–20	Oct. 16–Nov. 2	50	1	100	Oct. 16–25: 21°–23° Oct. 26–29: 23°–25° Oct. 30–Nov. 2: 19°–22°	*B*
247a × *247a*.....	*E*	Dec. 9–15	Dec. 9–27	97	1	194	21°–23°	*A*
E40a × *E40a*.....	*F*	Jan. 12 1931	Jan. 12–25	48	1	96	17°–29°, but usually 21°–26°	*A*
E41a × *E41a*.....	*G*	Jan. 12–13	Jan. 12–25	48	1	96	ditto	*A*
E85b × *E85b*.....	*H*	Jan. 12–13	Jan. 12–25	48	1	96	ditto	*A*
E81a × *E81a*.....	*J*	Jan. 12–14	Jan. 12–25	48	1	96	ditto	*A*
E80b × *E80b*.....	*K*	Jan. 13–14	Jan. 13–25	48	1	96	ditto	*A*
E46b × *E46b*.....	*L*	Jan. 12–14	Jan. 12–25	48	1	96	ditto	*A*
E81a × *E81a*.....	*M*	Feb. 9–12	Feb. 9–22	44	1	88	23°–25°	*A*
E40a × *E40a*.....	*P*	Feb. 11	Feb. 11–14	48	1	96	23°–25°	*A*
E81a × *E81a*.....	M_1	Feb. 15–18	Feb. 15–28	96	1	192	23°–25°	*A*
E40a × *E40a*.....	P_1	Feb. 15–18	Feb. 15–28	48	1	96	23°–25°	*A*
E40a × *E40a*.....	*S*	Mar. 11–16	Mar. 11–Apr. 1	20	2	80	20.5°–28.5°, but usually 21°–26°	*C*
E81a × *E81a*.....	*T*	Mar. 11–16	Mar. 11–Apr. 1	21	2	84	ditto	*C*
E40a × *E81a*.....	*X*	Mar. 11–15	Mar. 11–Apr. 1	20	2	80	ditto	*C*
Total: *247a* × *247a*				205		410		
Total: *E40a* × *E40a*				164		348		
Total: *E81a* × *E81a*				209		439		

useful information about them, in Table I. There are given, in column 1, the names of the clones of the two conjugating individuals; in column 2, the designation given to each particular group of ex-conjugants; in column 3, the dates on which conjugation occurred; in column 4, the dates between which the ex-conjugants were studied; in column 5, the number of pairs of conjugants in each group; in column 6, the number of lines of descent from each ex-conjugant; in column 7, the total number of lines in each group; in column 8, the temperature range during each experiment; in column 9, the culture method employed.

Figure 1 and Table I are intended to give the general plan of the investigation and many of the pertinent details. At the beginning of each of the subsequent sections of the paper it may be helpful to refer both to Fig. 1 and to Table I.

III. Three Conjugations within the Clone 247*a*

Most of the necessary information concerning the origin of clone 247*a* and the details of the three conjugations within it have been given in the preceding sections. (See especially Fig. 1 and Table I.) With descendants of the 58 pairs of conjugants obtained on October 1–2, the twenty-four co-existing non-conjugant lines were compared during two successive five-day periods (December 18–22 and December 23–27).

Table II gives a detailed picture of the distribution of total numbers of fissions obtained in these experiments, and shows some relations that will be more precisely formulated by statistical methods in other tables. It is at once apparent that the range of fission rates of the ex-conjugants in every period exceeds that of the non-conjugants. Less obvious is the fact that the values for the ex-conjugants are less concentrated within the modal range than are the values for the non-conjugants. Calculations based on Table II show that on the average, only 73.3 per cent of the ex-conjugant values for five-day periods are concentrated in the three most frequent classes of values, whereas 93.1 per cent of the non-conjugant values are within their three most frequent classes.

Table II also shows clearly that the numbers of fissions in five days vary greatly from period to period in both groups. Taking the modal values as an illustration, the ex-conjugant mode drops from 11 fissions in the first period to 5 in the second period of the first experiment. The non-conjugant mode dropped from 10 to 6 during the same periods. These two periods are typical of the sort of changes that were observed in many experiments. At the time of such shifts of mode in one group, similar changes were observed in the reproductive rates of all the races being cultivated, so that it appeared practically certain that the change was due to some change in the conditions of cultivation.

TABLE II

Comparison of non-conjugants of clone 247a with three sets (C, D and E) of ex-conjugants derived from them at different times. Distribution of total number of fissions during five-day periods.

Five-day periods	Group	Total number of fissions													Number of lines
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	
Exp. 1. Oct. 5–9, 1930..	Ex-conjugant group *C*					1	1		1	4	8	19	35	21	5	
	Non-conjugants of 247*a*										3	12	7			
Oct. 10–14, 1930.....	Ex-conjugant group *C*	2	2	1	9	19	30	18	6	1	2					
	Non-conjugants of 247*a*		1		1		3	9	5							
Exp. 2. Oct. 24–28, 1930	Ex-conjugant group *D*	3	1	2		1	3	4	4	6	15	17	13	1		
	Non-conjugants of 247*a*			1						2	5	5	1			
Oct. 29–Nov. 2, 1930 ..	Ex-conjugant group *D*		1	1	8	17	10	3	8	2	1	1				
	Non-conjugants of 247*a*				1	5	5	2								
Exp. 3. Dec. 18–22, 1930	Ex-conjugant group *E*		2	3	1	5	1	3	1	3	13	65	73	7		
	Non-conjugants of 247*a*							1	1	3	8	9				
Dec. 23–27, 1930.....	Ex-conjugant group *E*	2	11	4	1	1	2	2	7	5	23	35	58	15	1	
	Non-conjugants of 247*a*									4	9	8				

TABLE III

Comparison of non-conjugants of clone 247a with three sets (C, D, and E) of ex-conjugants derived from them at different times. Mean total numbers of fissions, with their standard deviations and coefficients of variation.

Experiment	Five-day periods	Group	Mean total number of fissions	Standard deviation	Coefficient of variation
1	Oct. 5–9, 1930.....	Ex-conjugant group *C*	10.65 ± 0.11	1.51 ± 0.07	14.21 ± 0.71%
		247*a* non-conjugants	10.18 ± 0.09	0.65 ± 0.07	6.38 ± 0.65%
	Oct. 10–14, 1930.....	Ex-conjugant group *C*	4.81 ± 0.11	1.58 ± 0.08	32.92 ± 1.83%
		247*a* non-conjugants	5.68 ± 0.22	1.45 ± 0.16	25.56 ± 2.97%
2	Oct. 24–28, 1930.....	Ex-conjugant group *D*	8.34 ± 0.23	2.89 ± 0.17	34.61 ± 2.20%
		247*a* non-conjugants	8.86 ± 0.37	2.07 ± 0.26	23.32 ± 3.13%
	Oct. 29–Nov. 2, 1930.....	Ex-conjugant group *D*	4.89 ± 0.17	1.79 ± 0.12	36.72 ± 2.74%
		247*a* non-conjugants	4.62 ± 0.16	0.84 ± 0.11	18.11 ± 2.47%
3	Dec. 18–22, 1930.....	Ex-conjugant group *E*	9.83 ± 0.11	2.11 ± 0.08	21.44 ± 0.80%
		247*a* non-conjugants	9.05 ± 0.15	1.07 ± 0.11	11.77 ± 1.21%
	Dec. 23–27, 1930.....	Ex-conjugant group *E*	9.13 ± 0.16	3.12 ± 0.12	34.12 ± 1.40%
		247*a* non-conjugants	9.19 ± 0.11	0.73 ± 0.08	7.96 ± 0.83%
	Ten-day periods				
1	Oct. 5–14, 1930.....	Ex-conjugant group *C*	15.70 ± 0.16	2.30 ± 0.12	14.68 ± 0.75%
		247*a* non-conjugants	15.78 ± 0.29	1.84 ± 0.21	11.68 ± 1.33%
2	Oct. 24–Nov. 2, 1930.....	Ex-conjugant group *D*	13.87 ± 0.27	2.89 ± 0.19	20.84 ± 1.44%
		247*a* non-conjugants	13.74 ± 0.21	1.05 ± 0.15	7.67 ± 1.11%
3	Dec. 18–27, 1930.....	Ex-conjugant group *E*	19.26 ± 0.23	4.37 ± 0.16	22.67 ± 0.88%
		247*a* non-conjugants	18.32 ± 0.17	1.08 ± 0.12	5.89 ± 0.76%

It is a remarkable fact that in spite of the great shifts of mode from period to period, the modes of the ex-conjugants of *247a* and the modes of the non-conjugants of this clone were always very similar within any one period. Such a persistent similarity in modes appears in Table II. During the first experiment, the modes of ex-conjugants (using ex-conjugant group *C*) and non-conjugants are 11 and 10 for the first period, 5 and 6 for the second period; during the second experiment (using ex-conjugant group *D*) the modes are 10 and 9–10 for the first period, 4 and 4–5 for the second period; during the third experiment (using ex-conjugant group *E*) the modes are 11 and 10 for the first period, 11

TABLE IV

Ratio of ex-conjugant groups (C, D, and E) to non-conjugant groups of their parent clone (247a) in mean total number of fissions, standard deviations, and coefficients of variation. These ratios are calculated from the data in Table III.

Ten-day periods	Experiment	Ex-conjugant groups compared with groups of non-conjugants of 247a	Ratio of ex-conjugant group to synchronous group of non-conjugants of 247a		
			in mean total number of fissions	in standard deviation	in coefficient of variation
Oct. 5–14.....	1	C	1.0	1.3	1.3
Oct. 24–Nov. 2....	2	D	1.0	2.8	2.7
Dec. 18–27.....	3	E	1.1	4.1	3.9

and 9 for the second period. This close agreement of modal values of non-conjugant and ex-conjugant groups within the same period, in spite of great fluctuation in mode from period to period, must be due to some innate similarity between these two kinds of groups, both derived originally from the same ancestors.

Tables III and IV demonstrate statistically the relations which Table II showed on inspection. In Table III the mean fission rate with its standard deviation and its coefficient of variation are given for both ex-conjugant and non-conjugant groups for all the periods of the experiments. In Table IV, the ratios of each of these statistical values for the ex-conjugant group to the corresponding values for the non-conjugant group are given. Again appears in Table III the great fluctuation in means from period to period. The ex-conjugant mean drops from 10.65 to 4.81 in the two periods of the first experiment and from 8.34 to 4.89 in the two periods of the second experiment. Also the means of the two groups in every period again appear remarkably similar: 10.65 and 10.18; 4.81 and 5.68; 8.34 and 8.86; 4.89 and 4.62; 9.83 and 9.05; 9.13 and 9.19. In three of the six periods the ex-conjugant mean

exceeds the non-conjugant mean by an average of 0.51 fission; in the other three periods the ex-conjugant mean is less than the non-conjugant mean by an average of 0.48 fission. Thus the algebraic mean difference between the five-day means for the two groups is practically negligible. This similarity between the means of ex-conjugants and non-conjugants of the same race is expressed another way in Table IV where the ratio of the former to the latter is given. The ratios for the three experiments (total of ten days each) are 1.0, 1.0 and 1.1. The uniformity of this ratio of ex-conjugant mean to non-conjugant mean in all three experiments is the more remarkable because of the large differences in the absolute values of the means for the three experiments (Table III). In the first experiment the values are 15.70 and 15.78, in the second experiment 13.87 and 13.74; in the third experiment, 19.26 and 18.32. It is therefore quite clear that in the clone *247a* the mean value of the fission rate is not changed by conjugation; nevertheless it should be remembered that some of the individual values, as well as the distribution of values, among the ex-conjugant groups are very different from those of the non-conjugant groups. So far as mean fission rate is concerned, there is in these experiments neither rejuvenescence nor depression as a result of conjugation.

For the purposes of the present paper, this is the most important result of these three experiments. But it should be remembered that so far as the separate lines are concerned, many ex-conjugants are very diverse from the non-conjugants. In the first experiment one ex-conjugant line reproduced more slowly than any non-conjugant line (13 others reproduced more slowly than all non-conjugants but one), 5 more rapidly, and 84 at the same rates as the non-conjugants; in the second experiment, 10 ex-conjugant lines reproduced more slowly than any non-conjugant line, 11 more rapidly, and 31 at the same rates as the non-conjugants; in the third experiment, 22 ex-conjugant lines reproduced more slowly than any non-conjugant lines, 95 more rapidly, and 50 at the same rates as the non-conjugants. Thus, in all three experiments, conjugation produced some rejuvenated lines, some depressed lines, and many lines that were unaltered in fission rate. It is only when all types of lines are averaged together that no effect of conjugation on fission rate appears. In this *average* absence of effect, all three experiments are in complete agreement.

The effect of conjugation on variability of fission rate has already been indicated. Table III shows that both the absolute variability, as measured by the standard deviation, and relative variability, as measured by the coefficient of variation, are greater for the ex-conjugants than for the non-conjugants, in every period of all three experiments. Conjuga-

tion has unmistakably increased the variation, both relative and absolute, of the fission rate. This result is in agreement with the results of Jennings (1913), Raffel (1930), and Jennings *et al.* (1932). In Table IV are given the ratios of the ex-conjugant standard deviations and coefficients of variation to the corresponding values for the non-conjugants. Because of the close agreement of the means of corresponding groups of ex-conjugants and non-conjugants, little difference appears between the ratios of the standard deviations and the ratios of the coefficients of variation in any one experiment. The two ratios differ very much, however, from experiment to experiment. For the first experiment both ratios are 1.3; for the second experiment, the ratios are 2.8 and 2.7, respectively; for the third experiment, 4.1 and 3.9, respectively. Thus conjugation has increased both relative and absolute variation in fission rate in all three experiments. In each case it has given rise to lines with higher fission rate, and lines with lower fission rate, as well as to lines with unchanged fission rate.

As to the relative mortality of conjugants and non-conjugants, no constant result was found in these three experiments. In the first experiment the mortality was 22.4 per cent for the ex-conjugant lines, 20.8 per cent for the non-conjugant lines; in the second experiment, 44.1 per cent for the ex-conjugants, 31.2 per cent for non-conjugants; in the third experiment, 10.2 per cent for ex-conjugants, 20.8 per cent for non-conjugants. Thus the mortality is nearly alike in the two groups in the first experiment, it is greater among the ex-conjugants in the second experiment, and is greater among the non-conjugants in the third experiment.

The three experiments on the effects of conjugation in the race 247*a* have thus shown, as far as they go, that conjugation does not always produce the same effect on mortality in this race, but that it does always produce the same effect on mean fission rate, on absolute variability, and on relative variability. The mean fission rate is regularly unchanged by conjugation, so that conjugation neither rejuvenates nor depresses the population as a whole. However, both absolute variability and relative variability of fission rate are regularly increased by conjugation. This results, in spite of absence of the effect on the mean, in the regular production of both depressed and rejuvenated ex-conjugants.

IV. Simultaneous Conjugations within Six Ex-conjugant Clones Derived from the Clone 247*a*

The preceding section brought out the fact that in clone 247*a*, although the *relations* of ex-conjugants to non-conjugants remained the same in different periods, the *absolute values* for both ex-conjugants and

non-conjugants were very different in different periods. This showed the desirability of conducting experimental comparisons between different clones cultivated in the same medium at the *same time*. Therefore, in an attempt to discover whether conjugation produces different effects in different clones, we compared the effects of *simultaneous* conjugations in *six* clones. To make the issue sharper, we deliberately selected six closely related clones that had similar characteristics. These were six of the most rapidly reproducing sister ex-conjugant clones (*E*40*a*, *E*41*a*, *E*46*b*, *E*80*b*, *E*81*a* and *E*85*b*) produced in the *E* conjugation of the clone 247*a* (see Fig. 1). The conjugations induced in them on January 12–14 (see Table I) were designated the *F*, *G*, *L*, *K*, *J*, and *H* conjugations, respectively. Records of fission rates and mortality among the conjugants and corresponding non-conjugants were kept until January 25.

From these data an answer to the question of the possible difference in effect of conjugation in different clones may be sought in two ways. On the one hand, the groups of ex-conjugants of the six clones may be compared as to fission rate, variability and mortality. On the other hand, the ex-conjugants of each clone may be compared with the non-conjugants of the same clone. For the latter comparison the data are not so extensive as could be desired, since it was impossible, for technical reasons, to keep in progress a large number of non-conjugant lines at the same time with the numerous ex-conjugant lines. But experience has shown that a small sample of non-conjugants usually gives a fair picture of the general character of the non-conjugant population, there being, as a rule, little variation among the members of a single clone multiplying vegetatively. On this account we feel that the comparison of the ex-conjugants with the small group of 6 or 8 lines of non-conjugants from the same clone is of interest, though of less value than would be the case if the non-conjugant groups were larger.

We take up first the comparison of the groups of ex-conjugants from the six clones. Table V gives the distribution of total number of fissions in two five-day periods (January 16–20 and 21–25) among the ex-conjugants from each clone. From these data may be calculated means, standard deviations and coefficients of variation of fission rate; these are given in Table VI. Among the six groups of ex-conjugants there are three (*E*41*a*, *E*80*b*, and *E*81*a*) which have consistently different mean fission rates in both five-day periods. *E*41*a* is consistently highest, *E*80*b* consistently intermediate, and *E*81*a* consistently lowest. Their mean fission rates are 16.69, 16.38, and 7.46 for the first period, 13.66, 13.26 and 5.39 for the second period. The differences between

TABLE V

Comparison of groups of non-conjugants of six clones (E40a, E41a, E46b, E80b, E81a, E85b) with the ex-conjugant groups derived from them. Distribution of total numbers of fissions in two five-day periods.

Clone	Period	Group	Total number of fissions																				
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E40a	Jan. 16–20....	Ex-conjugants											1				9	13	24	21	18	1	
		Non-conjugants														3	1	3	1				
	Jan. 21–25....	Ex-conjugants								1	1	1	1	1	7	18	27	22	5				
		Non-conjugants											1		1	3	1	2					
E41a	Jan. 16–20....	Ex-conjugants						1	1	1		1			1	1	3	7	8	23	25	13	1
		Non-conjugants																1	2	3	2		
	Jan. 21–25....	Ex-conjugants			1	1			1		3		2	1	1	12	23	35	5				
		Non-conjugants												1	1	1	3	2					
E46b	Jan. 16–20....	Ex-conjugants					1	1		1		1		2		2	4	6	13	22	18	7	1
		Non-conjugants															1		1	3	1		
	Jan. 21–25....	Ex-conjugants						2			1		1	4	3	8	24	21	6				
		Non-conjugants										1		1	2	1	1	1					
E80b	Jan. 16–20....	Ex-conjugants							1			3		2				5	19	23	17	6	
		Non-conjugants												1			1	3	2				
	Jan. 21–25....	Ex-conjugants			2	1	1		1			1	1	3	1	5	32	23	1				
		Non-conjugants												1	2	1		2					
E81a	Jan. 16–20....	Ex-conjugants	2	1	4		2	2	6	3	6	3	3	2	2	2	1	1	1				
		Non-conjugants												1	1	1	2		2				
	Jan. 21–25....	Ex-conjugants	1	3	1		3	2	2	1	1	1	2				1						
		Non-conjugants						2		1		1				1	1						
E85b	Jan. 16–20....	Ex-conjugants	1	2					2		1	1	1		2		3	6	11	28	9	4	
		Non-conjugants															1	1	3	1			
	Jan. 21–25....	Ex-conjugants						1	1					3	2	10	22	18	3				
		Non-conjugants											1			1	2	3					

TABLE VI

Comparison of groups of non-conjugants of six clones (E40a, E41a, E46b, E80b, E81a, E85b) with groups of ex-conjugants derived from them. Mean total numbers of fissions, with their standard deviations and coefficients of variation.

Period	Clone	Mean total number of fissions		Standard deviations		Coefficients of variation	
		Ex-conj.	Non-conj.	Ex-conj.	Non-conj.	Ex-conj.	Non-conj.
Jan. 16–20, 1931.....	*E40a*	16.26 ± 0.10	14.25 ± 0.26	1.44 ± 0.07	1.09 ± 0.18	8.87 ± 0.46%	7.65 ± 1.30%
	E41a	16.69 ± 0.19	16.75 ± 0.23	2.66 ± 0.14	0.97 ± 0.16	15.97 ± 0.84%	5.78 ± 0.98%
	E46b	16.18 ± 0.22	16.50 ± 0.35	2.88 ± 0.16	1.26 ± 0.25	17.81 ± 0.99%	7.63 ± 1.49%
	E80b	16.38 ± 0.19	14.57 ± 0.41	2.40 ± 0.13	1.59 ± 0.29	14.62 ± 0.82%	10.92 ± 1.99%
	E81a	7.46 ± 0.42	13.71 ± 0.45	3.98 ± 0.30	1.75 ± 0.32	53.38 ± 4.98%	12.76 ± 2.34%
	E85b	15.31 ± 0.33	15.67 ± 0.26	4.06 ± 0.23	0.94 ± 0.18	26.53 ± 1.60%	6.02 ± 1.18%
Jan. 21–25, 1931.....	*E40a*	13.70 ± 0.12	13.13 ± 0.37	1.60 ± 0.08	1.54 ± 0.26	11.69 ± 0.62%	11.70 ± 2.00%
	E41a	13.66 ± 0.18	13.50 ± 0.32	2.52 ± 0.14	1.32 ± 0.22	18.44 ± 1.09%	9.80 ± 1.67%
	E46b	13.70 ± 0.17	12.29 ± 0.47	2.10 ± 0.12	1.83 ± 0.33	15.33 ± 0.89%	14.89 ± 1.98%
	E80b	13.26 ± 0.24	13.00 ± 0.42	2.98 ± 0.17	1.53 ± 0.30	22.44 ± 1.32%	11.75 ± 2.32%
	E81a	5.39 ± 0.59	8.83 ± 0.99	3.68 ± 0.41	3.58 ± 0.70	68.36 ± 10.69%	40.51 ± 9.09%
	E85b	13.73 ± 0.17	13.71 ± 0.43	1.90 ± 0.12	1.67 ± 0.30	13.81 ± 0.87%	12.15 ± 2.22%

E41a and *E81a* are 9.23 ± 0.45 for the first period and 8.27 ± 0.62 for the second period. The differences between *E80b* and *E81a* are 8.92 ± 0.45 for the first period and 7.87 ± 0.64 for the second period. The differences between *E41a* and *E80b* are 0.31 ± 0.27 for the first period and 0.40 ± 0.30 for the second period. Thus the differences between *E41a* and *E81a*, and the differences between *E80b* and *E81a* are clearly significant, the differences ranging from twelve to twenty times their probable errors. On the other hand, the differences between *E41a* and *E80b* are only 1.1 to 1.3 greater than their probable errors and therefore cannot be considered as certainly significant. The uniformity of these differences between *E41a* and *E80b*, however, makes them somewhat more significant than their probable errors would indicate. It is clear, in any case, that there is a well-defined difference in fission rate between *E81a* and all the other groups. The differences between *E81a* and the

TABLE VII

Differences in standard deviation among the three grades of ex-conjugant groups. Grade 1 = group *E40a;* grade 2 = groups *E41a*, *E46b*, and *E80b;* grade 3 = group *E81a*.

Grades Compared	Groups Compared	Period 1		Period 2	
		Difference	Difference / P.E.	Difference	Difference / P.E.
3–2....	*E81a–E46b*	1.10 ± 0.34	3.2	1.58 ± 0.43	3.7
3–2....	*E81a–E41a*	1.32 ± 0.33	4.0	1.16 ± 0.43	2.7
3–2....	*E81a–E80b*	1.58 ± 0.33	4.8	0.70 ± 0.44	1.6
2–1....	*E41a–E40a*	1.22 ± 0.16	7.6	0.92 ± 0.16	5.8
2–1....	*E46b–E40a*	1.44 ± 0.18	8.0	0.50 ± 0.14	3.6
2–1....	*E80b–E40a*	0.96 ± 0.14	6.9	1.38 ± 0.19	7.3
3–1....	*E81a–E40a*	2.54 ± 0.31	8.2	2.08 ± 0.42	5.0

group nearest it in each period are 7.85 ± 0.53 (*E85b–E81a*) in the first period and 7.87 ± 0.64 (*E80b–E81a*) in the second period.

In absolute variability, Table VI shows that the six ex-conjugant groups fall into three consistently distinct grades: one with standard deviation near 1.5 fissions, one between 2.0 and 3.0 fissions, and one between 3.7 and 4.0 fissions, for the two five-day periods. The group *E40a* had standard deviations of 1.44 and 1.60 fissions; these were lower than those of any other group. The groups *E41a*, *E46b*, and *E80b* all fell in an intermediate grade with standard deviations varying from 2.0 to 3.0 fissions and without consistent differences among the three groups in this grade. The group *E81a*, with standard deviations

of 3.98 and 3.68 fissions had a variation distinctly higher than any other group.

Not only are the differences between these three grades of standard deviations consistent, but they are also statistically significant, as appears in Table VII. All the differences except those in the second period between *E41a* and *E81a* and between *E80b* and *E81a* are more than three times their probable error. Therefore the differences between the three groups *E40a, E46b,* and *E81a* are clearly significant and establish the existence of three grades of standard deviations among the six races.

The data on relative variability of fission rate, as measured by the

TABLE VIII

Differences in coefficients of variation among the three grades of ex-conjugant groups. Grade 1 = group *E40a;* grade 2 = groups *E41a, E46b,* and *E80b;* grade 3 = group *E81a.*

Grades Compared	Groups Compared	Period 1		Period 2	
		Difference	Difference / P.E.	Difference	Difference / P.E.
3–2....	*E81a–E80b*	38.76 ± 5.05	7.7	45.92 ± 10.77	4.3
3–2....	*E81a–E46b*	35.57 ± 5.08	7.0	53.03 ± 10.72	5.0
3–2....	*E81a–E41a*	37.41 ± 5.05	7.4	49.92 ± 10.74	4.7
2–1....	*E80b–E40a*	5.75 ± 0.94	6.1	10.75 ± 1.46	7.4
2–1....	*E46b–E40a*	8.94 ± 1.01	8.9	3.64 ± 1.08	3.4
2–1....	*E41a–E40a*	7.10 ± 0.96	7.4	6.75 ± 1.21	5.6
3–1....	*E81a–E40a*	44.51 ± 5.00	8.9	56.67 ± 10.71	5.3

coefficient of variation are also given in Table VI. Differences in relative variability among the ex-conjugant groups closely parallel the differences in absolute variability. The group *E40a* is again the least variable with coefficients of 8.87 per cent and 11.69 per cent; *E41a, E46b,* and *E80b* again fall into the intermediate grade, with coefficients from 14.62 per cent to 22.44 per cent; and *E81a* is again the most variable, with coefficients of 53.38 per cent and 68.36 per cent. As appears in Table VIII, the differences between these three grades are clearly statistically significant in every case and in both periods.

The data on mortality, as measured by the ratio of the number of lines that died out during a given period to the number of lines that were alive at the beginning of the period can be expressed as percentages. These percentages, in the cases of the groups of ex-conjugants,

are the percentages of lines, or ex-conjugant clones (since only one line of each ex-conjugant clone was in progress), that died out during a given period. The percentages of mortality among the six groups of ex-conjugant clones, during the whole experiment of January 16–25, fell into three distinctly different grades.

The groups *E40a* and *E41a* had a very low mortality (12.5 per cent and 11.5 per cent, respectively); the groups *E46b*, *E80b* and *E85b* had a moderate mortality (35.7 per cent, 24.2 per cent, and 37.5 per cent, respectively); and the group *E81a* had a very high mortality (81.3 per cent). In these differences there appears some foundation for the conflicting results of different investigators concerning the effect of conjugation on mortality. *E40a* and *E41a* are clones of such genetic constitution that conjugation results in but a small proportion of non-viable combinations of nuclear material; on the other hand, *E81a* is a clone whose genetic constitution is such that, under the same conditions of experiment, a very high proportion of non-viable combinations result from conjugation. The question of the effect of conjugation on mortality cannot properly be asked in the form "What is the effect of conjugation on mortality?" for the answer will differ in different cases; the answer which will be discovered in any particular case depends upon the genetic constitution of the race under examination. In some races conjugation will result in very great mortality; in other races, in very little mortality.

In the preceding account of differences among the groups of ex-conjugants derived from six related clones, the most outstanding features were the great differences between ex-conjugants from the clone *E81a* and ex-conjugants from all the other clones. These differed in mean fission rate, in relative variability, in absolute variability, and in mortality. In dealing with Protozoa studied in daily isolation culture, the possibility must always be kept in mind that differences observed between the groups investigated might result from a diversity in the bacterial flora of the culture fluid such as might be perpetuated from day to day. Although we were always aware of this danger and, as a routine part of our experimental procedure, took numerous precautions to avoid it, we thought it best to make a direct test in the case of the clone *E81a*. The test consisted in retaining the twenty-one separate drops of the culture fluid in which twenty-one ex-conjugant lines from *E81a* had died and transferring to each of these drops a single specimen from one of the normal lines of one of the other groups of ex-conjugants. To each of these drops, one extra drop of fresh fluid was added, and the paramecia left overnight in the combination of old fluid and fresh fluid. Next day and thereafter, each line was treated in the

usual manner, being transferred daily to a drop of fresh culture fluid. As controls, we kept a sister animal of each of the paramecia put into the death-fluid of the *E*81*a* lines; these were cultivated in the usual culture medium, not mixed with the fluid in which the *E*81*a* ex-conjugants had died. The records of reproductive activity during the following five days for the test animals and controls were practically identical. It is certain, therefore, that the differences between the groups of ex-conjugants of *E*81*a* and the other ex-conjugant groups cannot be attributed to self-perpetuating differences in the conditions of cultivation. The only alternative is that the differences found are truly constitutional.

The phenomena observed among the non-conjugants of *E*81*a* give further evidence that this clone differed constitutionally from the others. The original *E*81*a* clone, as well as the other five clones, were selected for study because preliminary observations had shown them to be the most rapid reproducers that we had in the laboratory. The astonishing inviability and low fission rates of the *E*81*a* ex-conjugants was the first evidence that this race differed markedly from the others. Later, however, the non-conjugants themselves underwent a sudden and marked change of character. The mean fission rate dropped from about three divisions a day to less than two divisions a day. This abrupt change occurred in the non-conjugants about one week after other members of the clone had been allowed to conjugate. The difference between the ex-conjugants of *E*81*a* and the ex-conjugants of the other clones was apparent immediately after conjugation. The change thus appeared one week earlier in the ex-conjugants than in the non-conjugants. This, it seems, is further strong evidence that the clone *E*81*a* was constitutionally different from the other clones: such difference, brought to light first in ex-conjugants from this clone, later appeared in the non-conjugants themselves.

Comparison of the non-conjugants and the ex-conjugants of other clones also brings out a relation of much interest. The diversities in the fission rate among the three ex-conjugant groups *E*41*a*, *E*80*b*, and *E*81*a* (see p. 271 above), are paralleled by the diversities in fission rate among their three non-conjugant groups (see Table VI). For the first period, the mean number of fissions attained by *E*41*a* non-conjugants was 16.75; by *E*80*b* non-conjugants 14.57; by *E*81*a* non-conjugants 13.71. For the second period, the non-conjugant mean attained by *E*41*a* was 13.50; by *E*80*b*, 13.00; by *E*81*a*, 8.83. It is remarkable that it is not possible to select any two clones from among the six studied, in which the clone having the faster multiplying non-conjugants consistently has the slower multiplying ex-conjugants. On the other hand, it is possible to select six pairs of clones (each of the five others com-

pared with *E81a*, and *E41a* compared with *E80b*) in which both the non-conjugants and the ex-conjugants of the one consistently multiply more rapidly than the corresponding groups of the other. All of the parent non-conjugant clones except *E81a* were so similar in mean fission rate that it is surprising that even one pair of clones (*E41a* and *E80b*) could be selected with consistent differences from period to period. These results are similar to the results of Calkins (1919 and 1920), Woodruff and Spencer (1924), and Jennings *et al.* (1932), in that diversities in mean fission rate between different lines of descent

TABLE IX

Comparison of six clones in the ratios of ex-conjugants to non-conjugants in mean total number of fissions, standard deviation, and coefficient of variation

	Period	Clones					
		E40a	*E41a*	*E46b*	*E80b*	*E81a*	*E85b*
Mean total number of fissions ..	Jan. 16–20	1.14	1.00	0.98	1.12	0.54	0.98
	Jan. 21–25	1.04	1.01	1.12	1.02	0.61	1.00
Standard deviation	Jan. 16–20	1.32	2.74	2.29	1.51	2.27	4.32
	Jan. 21–25	1.04	1.91	1.15	1.95	1.03	1.14
Coefficient of variation	Jan. 16–20	1.16	2.76	2.33	1.34	4.18	4.41
	Jan. 21–25	1.00	1.88	1.03	1.91	1.69	1.14

within the same species are found to persist through conjugation, reappearing as similar diversities between the derived sets of ex-conjugants.

In Table IX are given the ratios of ex-conjugant mean fission rates, standard deviations, and coefficients of variation to the corresponding values for non-conjugants. The ratios of the mean fission rates fall into three classes: those less than one, those equal to one, and those greater than one. The ratios for the clone *E81a* are 0.54 for the first period, and 0.61 for the second period. The corresponding ratios for the clone *E41a* are 1.00 and 1.01; and for the clone *E80b* they are 1.12 and 1.02. Thus, the mean fission rate of the clone *E81a* has been markedly decreased by conjugation; the mean fission rate of the clone *E41a* has been unchanged by conjugation; and the mean fission rate of the clone *E80b* has been very slightly increased by conjugation. With respect to the effect of conjugation on mean fission rate, *E41a* is a clone similar to *247a*, as described above on page 268. This simultaneous comparison of the effects of conjugation in six clones demonstrates

that conjugation does not have the same effect on mean fission rate in all clones: in some clones, conjugation raised very slightly the mean fission rate (rejuvenescence); in other clones, it lowered the mean fission rate (depression); and in other clones it left the mean fission rate unaltered. It appears here again that *the effect of conjugation on mean fission rate depends on the nature of the clone which conjugates.*

The ratios of the standard deviations in the ex-conjugants to those in the non-conjugants (Table IX) show a number of consistent differences, but most of them are slight. In *E40a* the ratio is lower than in *E85b, E41a, E46b, E80b*; in *E81a* it is lower than in *E85b* and *E41a*; and in *E46b* it is lower than in *E41a*. The greatest consistent difference in ratio is between *E40a* and *E41a*. The ratios for the former are 1.32 and 1.04, for the two different periods; for the latter the corresponding ratios are 2.74 and 1.91. Thus conjugation increased absolute variability of fission rate about twice as much in the clone *E41a* as in the clone *E40a*.

TABLE X

Comparison of clone E40a with clone E41a in differences between non-conjugants and ex-conjugants in standard deviation and coefficient of variation of total number of fissions.

		Jan. 16–20	Jan. 21–25
Difference between non-conjugants and ex-conjugants in Standard Deviations	Clone *E40a*	0.33 ± 0.19	0.06 ± 0.27
	Clone *E41a*	1.69 ± 0.21	1.20 ± 0.26
Difference between non-conjugants and ex-conjugants in Coefficients of Variation	Clone *E40a*	$1.22 \pm 1.38\%$	$0.01 \pm 2.09\%$
	Clone *E81a*	$10.19 \pm 1.18\%$	$8.64 \pm 1.99\%$

The ratios of the coefficients of variation of the ex-conjugants to those of the non-conjugants (also in Table IX) show differences similar to the differences in the ratios of the standard deviations. *E40a* is consistently lowest, with ratios of 1.16 and 1.00; *E41a* is among the highest, with ratios of 2.76 and 1.88. Thus the relative variability of *E41a* has been about twice as much increased by conjugation as the relative variability of *E40a*.

These results are another instance of the diversity of the effects of conjugation in different clones. In the clone *E40a*, conjugation has not significantly increased either absolute or relative variability. On the other hand, in the clone *E41a*, conjugation has increased both absolute and relative variability by 100 per cent to 200 per cent.

TABLE XI

Comparison of non-conjugants of clone E40a with non-conjugants of clone E81a, and of groups of ex-conjugants of clone E40a with groups of ex-conjugants of clone E81a, in four different conjugations. Distribution of total number of fissions in five-day periods.

Experiment	Period	Clones	Total number of fissions in five-day periods in ex-conjugant groups																		
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Jan. 16–20	*E40a*											1				9	13	24	21	18	1
		E81a	2	1	4		2	2	6	3	6	3	3	2	2	2	1	1	1			
	Jan. 21–25	*E40a*								1	1	1	1	1	7	18	27	22	5			
		E81a	1	3	1		3	2	2	1	1	1	2				1					
2	Feb. 12–14	*E40a*	4	7	1	3	8	12	11	22	11	5										
		E81a	4	8	3	3	4	5	1	1	1											
3	Feb. 19–23	*E40a*				1	2	4		2	3	5	4	5	7	6	7					
		E81a	1	2	4	4	5	10	3	7	6	3		3	1	1	1					
	Feb. 24–28	*E40a*		1	1	4		1	1		2	2	9	3		3						
		E81a				1																
4	Mar. 18–22	*E40a*	1		1				1		1	1	5	3	11	2						
		E81a		1		1	2			1			1									
	Mar. 23–27	*E40a*						1		3		2	1	1	7	7						
		E81a						2						1								
	Mar. 28–Apr. 1	*E40a*						1				1		4	1	5	4	4	1			
		E81a							1		1						1					

TABLE XI—*Continued*

Experiment	Period	Clones	Total number of fissions in five-day periods in non-conjugant groups																		
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	Jan. 16–20	*E40a*														3	1	3	1			
		E81a												1	1	1	2		2			
	Jan. 21–25	*E40a*											1		1	3	1	2				
		E81a						2		1		1				1	1					
2	Feb. 12–14	*E40a*									1	4	3									
		E81a								1	5	10										
3	Feb. 19–23	*E40a*										1	1	2	1		2					
		E81a											2	1	1	2	4					
	Feb. 24–28	*E40a*										1	1									
		E81a				1	1	1				1										
4	Mar. 18–22	*E40a*	1						1				2	1	1	1	1					
		E81a		1	1	2	2		4	2	1											
	Mar. 23–27	*E40a*						1	3			1	1	1	1							
		E81a	1	1	2	3	3	2		1	1											
	Mar. 28–Apr. 1	*E40a*													1	4	4	1				
		E81a							3		2	3	1	1								

As appears in Table X, the diversities between ex-conjugants and non-conjugants in *E40a* are small and insignificant, but in *E41a* they are large and clear. Thus an experiment on *E40a* alone would have led to the conclusion that conjugation does not increase variation; but an experiment on *E41a* alone would have led to the conclusion that conjugation greatly increases variability. The present experiment shows that neither conclusion tells the whole truth. The effect of conjugation on variability, like its effect on mean fission rate, depends upon the nature of the race which conjugates. In either characteristic, some races will manifest one effect of conjugation, other races a different or even opposite effect.

Comparison of the percentage mortality of the ex-conjugants with the percentage mortality of the non-conjugants brings out the same types of relations. The non-conjugants of clone *E85b* had a mortality of 37.5 per cent which was exactly the same as the percentage mortality among the group of ex-conjugants from this clone. In clone *E80b*, the non-conjugants had a mortality of 25.0 per cent; the ex-conjugants, 24.2 per cent. Clearly conjugation produced practically no change in the mortality of clones *E80b* and *E85b*. On the other hand, the non-conjugants of clone *E81a*, like the non-conjugants of clone *E85b*, had a mortality of 37.5 per cent, but the ex-conjugants of clone *E81a* had a mortality of 81.3 per cent. Thus, as with mean fission rate and variability, in some clones (*e.g.*, *E81a*), conjugation greatly alters mortality, and in other clones leaves mortality unchanged.

The evidence thus far shows clearly that diversities in the effect of conjugation in different clones are due to constitutional diversities between the clones. It seems desirable to discover whether such diverse effects in different clones are constant. Will the same diversities recur in repeated conjugations? This matter was tested in two of the clones that were found to give diverse results of conjugation; it is fully discussed in the next section.

V. Repeated Comparisons of the Effects of Conjugations in the Two Clones *E40a* and *E81a*

Conjugations within *the Clones E40a and E81a*

In order to determine whether the diverse effects of conjugation in different clones occur regularly, four sets of conjugants were obtained and studied in clones *E40a* and *E81a*. These eight sets of conjugants were obtained two at a time, one from each of the two clones. In every case, of course, conjugation in the two clones occurred in lines that had not conjugated since the *E* conjugation.

Results of the four sets of conjugations were studied during the intervals: January 16–25, February 12–14, February 19–28, and March 18–April 1. The experiments, as usual, were designed to compare mortality and fission rates, and variability of fission rates. As will appear in detail below, mortality among the ex-conjugants from the clone *E*81*a* was so high that, although comparison of rates of mortality could be made for each of the four experiments, comparisons of fission rate, variability of fission rate, and the ratios of the data of ex-conjugants to the data of non-conjugants in these respects could not be made for four of the eight periods of the experiments. During the remaining four periods (January 16–20, January 21–25, February 12–14, February 19–23), however, there were 41, 18, 30, and 51 ex-conjugant lines of

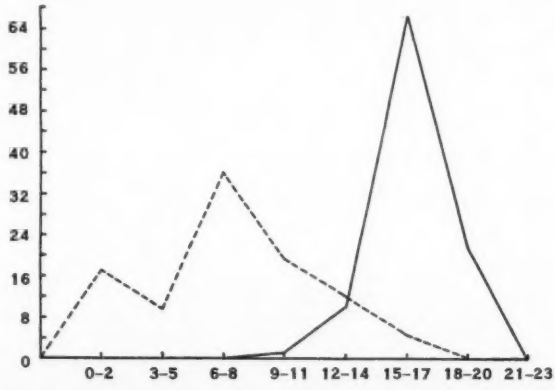

FIG. 2. Comparison of group of ex-conjugants from clone *E*40*a* (solid curve) with a group of ex-conjugants from *E*81*a* (broken curve), during the five-day period, January 16–20. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

*E*81*a* to compare with 87, 84, 84, and 46 ex-conjugant lines of *E*40*a*, so that statistical comparisons of fission rate and variability could be made.

The basic data for all eight periods of these four experiments are given in Tables XI and XIII. Table XI gives the basic data on fission rates, from which all the data on means, standard deviations and coefficients of variation are derived. Table XIII gives the data on mortality.

The most striking fact shown in Table XI is the difference between the distribution of values for the ex-conjugants of *E*81*a* and the distribution of values for the ex-conjugants of *E*40*a*. The former are

obviously massed considerably to the left of (that is, are lower in value than) the latter. This difference in distribution is brought out graphically by Figs 2, 3, 4, and 5; these are graphs on which are plotted the percentage of ex-conjugants attaining each number of fissions during a single five-day period (three-day period in Fig. 4). Figures 2 and 3 give the curves for the two periods of the first experiment; Fig. 4 gives the curves for the short second experiment; Fig. 5, the curves for the first period of the third experiment. In all periods of the fourth experiment too few ex-conjugants of *E81a* survived to warrant plotting curves.

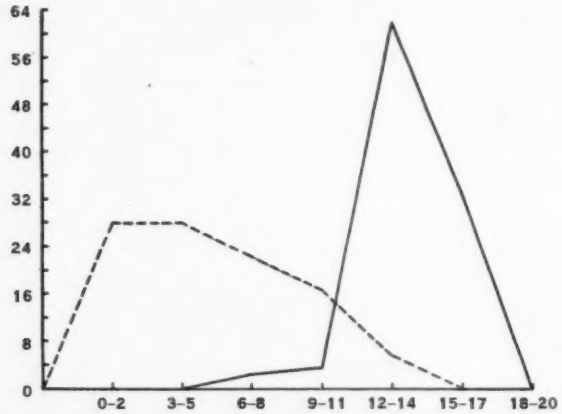

FIG. 3. Comparison of group of ex-conjugants from clone *E40a* (solid curve) with a group of ex-conjugants from *E81a* (broken curve), during the five-day period, January 21–25. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

In the first experiment (Figs. 2 and 3), the curves for the two groups of ex-conjugants are totally different. The solid lines, representing the groups of ex-conjugants from *E40a,* are curves peaked sharply between 15 and 17 fissions in the first period (Fig. 2) and between 12 and 14 fissions in the second period (Fig. 3). On the other hand, the broken lines, representing the groups of ex-conjugants from *E81a,* are long, low, and irregular, extending from 0 to 17 in the first period (Fig. 2), and from 0 to 14 in the second period (Fig. 3). The curves thus bring out the much greater range and variation, but much greater frequency of lower fission rates in the *E81a* group as compared with the *E40a* group.

In both the second experiment and the first period of the third experiment (Figs. 4 and 5), the curves for the two groups have nearly the

same range, but the curves for *E40a* are peaked near their upper limits and the curves of *E81a* near their lower limits. In the second experiment (Fig. 4) the peak of the *E40a* curve is between 6 and 8; the peak of the *E81a* curve between 0 and 2. In the first period of the third experiment (Fig. 5), the peak of the *E40a* curve is at 12–14 fissions;

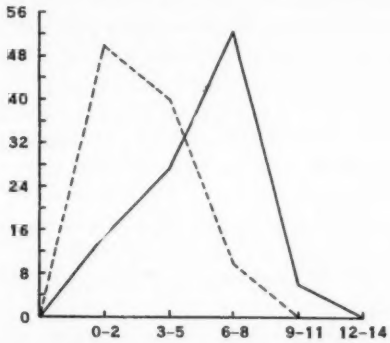

FIG. 4. Comparison of group of ex-conjugants from clone *E40a* (solid curve) with a group of ex-conjugants from *E81a* (broken curve), during the three-day period, February 12–14. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

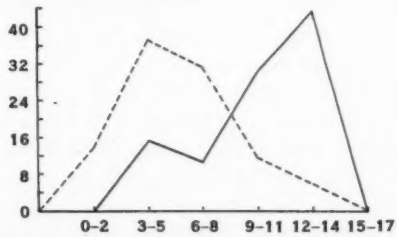

FIG. 5. Comparison of group of ex-conjugants from clone *E40a* (solid curve) with a group of ex-conjugants from *E81a* (broken curve), during the five-day period, February 19–23. The percentage of lines of each group (ordinate) is plotted against the number of fissions (abscissa).

the peak of the *E81a* curve at 3–5 fissions. These curves bring out the much more frequent occurrence of low fission rates among the *E81a* ex-conjugants as compared with the *E40a* ex-conjugants; and also the very similar range and absolute variation. The relative variability, nevertheless, is much greater among the *E81a* groups of ex-conjugants on account of their much lower means. Both of these sets of curves (Figs. 4 and 5) agree with the former sets (Figs. 2 and 3) in that the main portions of the curves for the *E40a* groups lie to the right of the main

portions of the corresponding *E81a* curves; that is, the majority of ex-conjugants from clone *E40a* have higher fission rates than the majority of ex-conjugants from clone *E81a*, during corresponding periods.

Table XII gives for all groups the mean fission rates, their standard deviations, and their coefficients of variation for all periods yielding statistically significant data. In every period the mean fission rate of the ex-conjugants of *E40a* is significantly higher than the mean fission rate of the ex-conjugants of *E81a*: 16.26 as compared with 7.46; 13.70 with 5.39; 5.51 with 2.83; 10.41 with 6.00. Thus the statistics on mean fission rate agree with the relation brought out graphically in Figs. 2, 3, 4, and 5; namely, that conjugation in the clone *E40a* uniformly yields groups of ex-conjugants with higher mean fission rates than does conjugation in the clone *E81a*.

The standard deviations do not show consistent significant differences between the two groups of ex-conjugants. In the first experiment, the *E81a* group has significantly greater standard deviations than the *E40a* group: 3.98 as compared with 1.44; and 3.68 as compared with 1.60. But in the second and third experiments the differences are not significant: 2.19 as compared with 2.12, and 3.13 as compared with 3.18. Again, the statistics show, in another way, the fact brought out in Figs. 2, 3, 4, and 5; namely, that the range in fission rate of biotypes produced by conjugation is not consistently different in the two groups.

The coefficients of variation, however, do show consistently significant differences. The coefficients of variation of the *E81a* groups of ex-conjugants are always greater than the coefficients of variation of the *E40a* groups of ex-conjugants: 53.38 per cent as compared with 8.87 per cent; 68.36 per cent as compared with 11.69 per cent; 77.38 per cent with 38.43 per cent; 52.08 per cent with 30.52 per cent. This regularly higher coefficient of variation in groups of ex-conjugants of clone *E81a*, together with the lack of consistent difference in standard deviations, can be due to but one thing: the lower mean fission rate of the *E81a* groups. This situation with respect to relative variability (coefficient of variation) and its dependence on differences in the means was indicated, it will be recalled, by Figs. 2, 3, 4, and 5, as mentioned above.

Table XIII gives the percentages of mortality in the different groups. In each experiment the mortality of the *E81a* group of ex-conjugants is very much greater than the mortality of the *E40a* group of ex-conjugants: 81.3 per cent as compared with 12.5 per cent; 40.9 per cent with 14.3 per cent; 99.5 per cent with 71.9 per cent; 92.9 per cent with 47.5 per cent. For the four experiments the average mortality among the groups of ex-conjugants from clone *E81a* was 81.3

TABLE XII

Comparison of groups of non-conjugants of clones E40a and E81a and of groups of ex-conjugants derived from each. Mean total number of fissions, with their standard deviations and coefficients of variation.

	Exp.	Period	Ex-conjugants		Non-conjugants	
			E40a	*E81a*	*E40a*	*E81a*
Mean total number of fissions.....	1	Jan. 16–20	16.26 ± 0.10	7.46 ± 0.42	14.25 ± 0.26	13.71 ± 0.45
		Jan. 21–25	13.70 ± 0.12	5.39 ± 0.59	13.13 ± 0.37	8.83 ± 0.99
	2	Feb. 12–14	5.51 ± 0.16	2.83 ± 0.27	9.25 ± 0.16	8.56 ± 0.10
	3	Feb. 19–23	10.41 ± 0.32	6.00 ± 0.30	11.57 ± 0.45	12.50 ± 0.34
Standard deviation.....	1	Jan. 16–20	1.44 ± 0.07	3.98 ± 0.30	1.09 ± 0.18	1.75 ± 0.32
		Jan. 21–25	1.60 ± 0.08	3.68 ± 0.41	1.54 ± 0.26	3.58 ± 0.70
	2	Feb. 12–14	2.12 ± 0.11	2.19 ± 0.19	0.66 ± 0.11	0.61 ± 0.07
	3	Feb. 19–23	3.18 ± 0.22	3.13 ± 0.21	1.76 ± 0.32	1.57 ± 0.24
Coefficient of variation.....	1	Jan. 16–20	8.87 ± 0.46%	53.38 ± 4.98%	7.65 ± 1.30%	12.76 ± 2.34%
		Jan. 21–25	11.69 ± 0.62%	68.36 ± 10.69%	11.70 ± 2.00%	40.51 ± 9.09%
	2	Feb. 12–14	38.43 ± 2.28%	77.38 ± 9.99%	7.15 ± 1.21%	7.12 ± 0.85%
	3	Feb. 19–23	30.52 ± 2.34%	52.08 ± 4.31%	15.22 ± 2.81%	12.52 ± 1.92%

per cent as compared with 36.1 per cent for the groups of ex-conjugants from clone *E40a*. There can be no doubt that conjugation regularly results in a very much greater mortality in the clone *E81a* than in the clone *E40a*.

Turning now to an examination of the relations between the values obtained for the ex-conjugants and the values obtained for the non-conjugants of the two races, the pertinent data will be found in Tables XIII and XIV, the former giving the mortality data, the latter giving the ratios of means, standard deviations, and coefficients of variation.

With one exception the ratios of ex-conjugant to non-conjugant mean fission rates in *E40a* are very close to 1.0, whereas the ratios in *E81a* vary from 0.33 to 0.61. The single exception in the case of *E40a* is in the second experiment. Observations in this experiment were dis-

TABLE XIII

Comparison of ex-conjugants and non-conjugants of clones E40a and E81a. Percentage mortality.

Experiment	Period	Clone *E40a*			Clone *E81a*		
		Ex-conj.	Non-conj.	Ex-conj. minus non-conj.	Ex-conj. minus non-conj.	Ex-conj.	Non-conj.
1	Jan. 12–25, 1931	12.5%	0 %	12.5%	43.8%	81.3%	37.5%
2	Feb. 9–14, 1931.....	14.3%	0 %	14.3%	40.9%	40.9%	0 %
3	Feb. 15–28, 1931	71.9%	75.0%	−3.1%	18.2%	99.5%	81.3%
4	Mar. 18–Apr. 1, 1931	47.5%	40.0%	7.5%	22.1%	92.9%	70.8%

continued after three days because of a defect in the culture method which resulted in great reduction in fission rates, and extraordinarily high mortality. For this reason, the data of this experiment are of doubtful value. The ratios in the other experiments were quite consistent: 1.14, 1.04, and 0.90 for *E40a*; 0.54, 0.61, and 0.48 for *E81a*. It therefore appears that, under favorable conditions, conjugation regularly makes little or no difference in the mean fission rate of *E40a*, but regularly reduces to about one-half the mean fission rate of *E81a*.

The ratios of standard deviations show no consistent differences between the two clones. On the other hand, the ratios of coefficients of variation are consistently higher in *E81a* than in *E40a*: 4.18 as compared with 1.16; 1.69 as compared with 1.00; 10.87 as compared with 5.38; 4.16 with 2.01. It is thus clear that conjugation regularly increases relative variability in the race *E81a* much more than it does in *E40a*. Within either race, the magnitude of the increase varies considerably in

different experiments, but within any one experiment the relative variability of *E81a* is increased more by conjugation than the relative variability of *E40a*. This is clearly due to the fact that conjugation greatly lowers the mean fission rate (without correspondingly reducing the standard deviation) in clone *E81a* but does not lower mean fission rate in clone *E40a*.

Conjugation also increases mortality by a greater amount in *E81a* than in *E40a*. In the first experiment the mortality of *E40a* is increased by conjugation from zero per cent to 12.5 per cent; a difference of 12.5 per cent; the mortality of *E81a* is increased from 37.5 per cent to 81.3 per cent, a difference of 43.8 per cent. In the second experi-

TABLE XIV

Comparison of clones E40a and E81a in the ratios of ex-conjugants to non-conjugants in mean total number of fissions, standard deviation and coefficient of variation

Experiment	Period	Mean total number of fissions		Standard deviation		Coefficient of variation	
		Clone *E40a*	Clone *E81a*	Clone *E40a*	Clone *E81a*	Clone *E40a*	Clone *E81a*
1	Jan. 16–20	1.14	0.54	1.32	2.27	1.16	4.18
	Jan. 21–25	1.04	0.61	1.04	1.03	1.00	1.69
2	Feb. 12–14	0.60	0.33	3.21	3.59	5.38	10.87
3	Feb. 19–23	0.90	0.48	1.81	1.99	2.01	4.16

ment, *E40a* increased from zero per cent to 14.3 per cent, a difference of 14.3 per cent; *E81a* increased from zero per cent to 40.9 per cent, a difference of 40.9 per cent. In the third experiment, *E40a* decreased from 75.0 per cent to 71.9 per cent, a decrease of 3.1 per cent; *E81a* increased from 81.3 per cent to 99.5 per cent, an increase of 18.2 per cent. In the fourth experiment, *E40a* increased from 40.0 per cent to 47.5 per cent, an increase of 7.5 per cent; *E81a* increased from 70.8 per cent to 92.9 per cent, an increase of 22.1 per cent.

Thus repeated comparison of the effects of conjugation within the two clones *E40a* and *E81a* confirms the diversity in effects of conjugation shown by the two clones in the experiments reported in Section IV and shows that differences of the same character occur at each conjugation. In clone *E81a* conjugation regularly decreases fission rates; while in clone *E40a* it does not. In clone *E81a* conjugation regularly increases the relative variability more than it does in *E40a* (this is a

consequence of the decrease in mean fission rate produced in *E81a*). Further, in *E81a*, conjugation regularly increases the mortality greatly, while in *E40a* it increases the mortality but slightly.

Conjugation between *the Clones E81a and E40a*

In view of the decided differences between these two clones, it appeared of great interest to attempt cross conjugation between them. This was done and twenty pairs of conjugants were obtained in which one member of each pair was from the clone *E40a*, the other member of the pair from the clone *E81a*. The ex-conjugant lines from these 40 individuals were compared with 40 ex-conjugants of *E40a* and with 40 ex-conjugants of *E81a*. The results of this experiment, together with the results of crosses between other diverse races, will be reported in detail later in a paper devoted exclusively to the subject of cross-conjugation. At this point, however, it may be stated that ex-conjugants of the cross between *E40a* and *E81a* differed markedly from non-conjugants of both clones and from ex-conjugants of both clones.

This result confirms the results previously elaborated in detail, namely, that the effects of conjugation on mean fission rate, on variability, and on mortality depend on the genetic constitution of the individuals which conjugate.

VI. Summary of Results and General Conclusions

Summary of results:

Part I.—Three conjugations within the clone *247a*:

1. Conjugation had no effect on mean fission rate in this clone.
2. Conjugation increased both standard deviation and coefficient of variation of fission rate.
3. Conjugation had no consistent effect on mortality in this clone.

Part II.—Simultaneous conjugations within six ex-conjugant clones derived from the clone *247a*:

1. In mean fission rate the ex-conjugants of one of the clones, *E81a*, are consistently and significantly lower than ex-conjugants of any of the other five clones.
2. In standard deviation, the ex-conjugants of clone *E81a* are consistently higher than those of all the other clones; those of *E40a* are consistently lower than all others; those of *E41a*, *E46b*, and *E80b* are consistently intermediate.
3. In coefficient of variation, the same significant consistent gradation of the groups exists as was found with respect to standard deviation.

4. In mortality, the ex-conjugants of clones *E40a* and *E41a* are very low, those of *E81a* very high, those of *E46b*, *E80b* and *E85b* intermediate.
5. It was demonstrated that the differences between the ex-conjugants of the clone *E81a* and those of the other clones were not due to self-perpetuating differences in cultural conditions; they were due to constitutional differences between this clone and the others.
6. Differences in mean fission rate between ex-conjugant groups parallel differences in mean fission rate between the non-conjugant groups from which they were derived.
7. The clone *E80b* had its mean fission rate very slightly increased by conjugation; the clone *E41a* had its mean fission rate unaltered by conjugation; the clone *E81a* had its mean fission rate reduced about 50 per cent by conjugation.
8. Conjugation increased absolute variability in fission rate about twice as much in the clone *E41a* as in the clone *E40a*.
9. Conjugation also increased the relative variability of fission rate about twice as much in the clone *E41a* as in the clone *E40a*.
10. Conjugation did not significantly increase variation in clone *E40a*, but did markedly increase it in clone *E41a*.
11. Conjugation produced practically no change in mortality in clones *E85b* and *E80b*, but it greatly increased mortality in the clone *E81a*.

Part III.—Four comparisons of the effects of conjugation in the clones *E40a* and *E81a*.

1. The distribution of fission rates among the ex-conjugants of the two clones are consistently different.
2. The mean fission rates of *E40a* ex-conjugants are consistently and significantly higher than the mean fission rates of *E81a* ex-conjugants.
3. The two groups of ex-conjugants do not differ consistently in standard deviation.
4. The coefficients of variation of the *E81a* groups of ex-conjugants are always greater than those of the *E40a* groups of ex-conjugants.
5. The mortality of *E81a* groups of ex-conjugants is always greater than the mortality of *E40a* groups of ex-conjugants.

6. Conjugation regularly leaves the mean fission rate of *E*40*a* unaltered, but regularly decreases markedly the mean fission rate of *E*81*a*.
7. Conjugation in these two clones produces no uniform differences in its effect on standard deviation.
8. Conjugation regularly increases the coefficient of variation in *E*81*a* more than in *E*40*a*.
9. Conjugation regularly increases mortality more in *E*81*a* than in *E*40*a*.
10. A group of cross-conjugants between the two diverse clones *E*81*a* and *E*40*a*, was found to differ from groups of non-conjugants and from groups of inbred conjugants of either one of the parent clones. (Details to be given in later paper.)

General Conclusions:

1. Conjugation increases fission rate in some clones, decreases fission rate in others; it increases variation in some clones, but (at least, under some conditions) not in others; it increases mortality in some clones, but not in others.
2. There are characteristic diversities between certain clones in the effect of conjugation on mean fission rate, variability, and mortality. These can be demonstrated repeatedly.
3. The physiological effects of conjugation are not the same in all clones of even a single species, but depend on the constitution of the individuals which conjugate, and differ when the constitutions of the conjugating individuals differ.
4. The diverse effects of conjugation found to be characteristic of different clones of *P. aurelia* bear an interesting relation to the diverse effects of conjugation (and the various theories of conjugation based on these diverse effects) reported by various investigators: Diversities similar to those reported to be characteristic of different species are here found to be characteristic of different clones of a single species. It is therefore suggested that the disagreement in results and theories of previous investigators may to some extent be due to failure to examine the effects of conjugation in a sufficient number of different races within each species. In *P. aurelia*, at least, results in agreement with each of the two leading theories of conjugation may be obtained by studying an appropriate race.

VII. BIBLIOGRAPHY

CALKINS, G. N., 1919. Uroleptus mobilis, Engelm. II. Renewal of vitality through conjugation. *Jour. Exper. Zoöl.*, **29**: 121.

CALKINS, G. N., 1920. Uroleptus mobilis, Engelm. III. A study in vitality. *Jour. Exper. Zoöl.*, **31**: 287.

JENNINGS, H. S., 1911. Assortative Mating, Variability, and Inheritance of Size, in the Conjugation of Paramecium. *Jour. Exper. Zoöl.*, **11**: 1.

JENNINGS, H. S., 1913. The Effect of Conjugation in Paramecium. *Jour. Exper. Zoöl.*, **14**: 279.

JENNINGS, H. S., 1929. Genetics of the Protozoa. *Bibliographia Genetica*, **5**: 105.

JENNINGS, H. S., AND K. S. LASHLEY, 1913. Biparental Inheritance and the Question of Sexuality in Paramecium. *Jour. Exper. Zoöl.*, **14**: 393.

JENNINGS, H. S., AND K. S. LASHLEY, 1913*a*. Biparental Inheritance of Size in Paramecium. *Jour. Exper. Zoöl.*, **15**: 193.

JENNINGS, H. S., D. RAFFEL, R. S. LYNCH, AND T. M. SONNEBORN, 1932. The Diverse Biotypes Produced by Conjugation in Paramecium aurelia. *Jour. Exper. Zoöl.*, (In press).

MACDOUGALL, MARY S., 1931. Another Mutation of Chilodon uncinatus Produced by Ultraviolet Radiation, with a Description of its Maturation Processes. *Jour. Exper. Zoöl.*, **58**: 229.

MAUPAS, E., 1888. Recherches expérimentales sur la multiplication des Infusoires ciliés. *Arch. d. Zool. Expér. et Gén.* (2), **6**: 165.

PASCHER, A., 1916. Über die Kreuzung einzelliger, haploider Organismen: Chlamydomonas. *Berichte Deutsch. Bot. Ges.*, **34**: 228.

RAFFEL, D., 1930. The Effect of Conjugation within a Clone of Paramecium aurelia. *Biol. Bull.*, **58**: 293.

RAFFEL, D., 1932. Inherited Variation Arising During Vegetative Reproduction in Paramecium aurelia. *Biol. Bull.*, **62**: 243.

WOODRUFF, L. L., AND H. SPENCER, 1924. Studies on Spathidium spathula. II. The significance of conjugation. *Jour. Exper. Zoöl.*, **39**: 133.

THE EFFECTS OF X-RAYS ON FERTILITY IN DROSOPHILA MELANOGASTER TREATED AT DIFFERENT STAGES IN DEVELOPMENT

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INTRODUCTION

Despite the numerous investigations in which *Drosophila* has been subjected to X-ray treatment, little is known concerning the effects which irradiation produces upon fertility in this fly. In 1927 Mavor employed the egg, larval, pupal, and adult stages of the fruit fly to determine the dosage of X-ray lethal to dipteran protoplasm. In 1926 and 1927 Packard reported the effects of X-rays upon protoplasm based upon the mortality of *Drosophila melanogaster* when subjected to X-ray treatment. In the treatment of mature males of *Drosophila,* Muller (1928) observed a high frequency of sterility in the adult offspring of the treated flies. Further work approaching the problem of the effects of irradiation upon fertility was accomplished by Hanson (1928). In this work mature male *Drosophila* were exposed to X-rays and the effects upon the mortality of eggs, larvæ and pupæ observed. A later report, and one more closely associated with fertility, is that of Hanson and Ferris (1929) upon fecundity in *Drosophila* exposed to irradiations.

A review of the literature, together with results obtained in a preliminary experiment in which eggs, larvæ and adults of both sexes were treated with X-rays, suggested that variations in fertility might be attributed to the variety of developmental stages of germ cells present at the time of treatment. This in turn suggested the problem of determining the effects of irradiation upon fertility when germ cells in different stages of development were subjected to X-ray treatment.

MATERIAL AND METHODS

In order to secure different stages of germ cells for treatment, wild type males were mated to wild type females. The flies were mated in vials which contained a small paper spoon filled with food. Females were allowed to deposit eggs for a period of 12 hours, after which the spoons were removed and others containing fresh food inserted into the vials. These second spoons were allowed to remain in the vials for an

additional 12 hours. Thus, all eggs deposited on the food were 0–12 hours old at the time of removal from the culture tubes. Egg bearing food from the spoons was placed in culture dishes to await the proper age for treatment. A portion of the eggs were held for 24 hours after the laying period was completed before treatment was given, a second portion for 72 hours; flies from a third portion were treated 30 hours after hatching and a fourth portion furnished males and females for the control series. Males and females from each group were separated upon emergence and held until nine days after treatment before they were mated. All cultures were kept at a temperature of 26.5° C. during development and all, except the controls, were given an equal amount of irradiation. The dosage of X-rays employed was that known as D5 (1325 *r* units), material being placed twelve centimeters from the target and the machine operated for twelve and one-half minutes at 50 kv. and 10 ma. using a one millimeter aluminum screen.

Matings for testing the fertility of the irradiated and control flies were made in vials containing spoons filled with food. In the case of treated individuals, a treated fly was mated to an untreated individual and if no eggs were deposited within two days after mating, the untreated individual of the pair was replaced by a second untreated fly. This precaution was deemed necessary to avoid errors due to the sterility of the untreated mate. Wild males and wild females were mated as controls.

Eggs were collected for a period of 8 days or until 100 eggs were secured from each mating. The spoons were removed from the tubes every twelve hours, the eggs counted under a binocular and the material transferred to food bottles where the eggs completed their development. Eggs were considered fertile only when they produced imagoes. Adults emerging from the eggs deposited by (or in the case of males fertilized by) an individual fly were counted and the percentage of fertility arrived at by dividing the number of adults by the original number of eggs in the culture. To obtain the percentage of fertility displayed by a group of flies, the mean fertility of the group was calculated by the formula

$$M = (a - c)i,$$

where a is an assumed mean, c is a correction factor and i the class interval, M being corrected by the probable error of the standard deviation of the group.

To determine the duration of the effects of irradiation upon fertility and sterility each group of flies was kept supplied with fresh food until twenty-five days had elapsed after the date on which they were treated

with X-rays. During this time both males and females (the same pair used in the 9 to 17-day period) were allowed to remain in the mating tubes. Beginning with the twenty-fifth day after irradiation eggs were collected over a period of 8 days, or until 100 eggs were obtained from each mating. Since a few of the treated flies failed to survive until the beginning of the second period, the comparisons of effects shown by the first collection of eggs (9 days after treatment) and those found in the second collection (25 days after treatment) involve slightly fewer flies than were considered in calculating the initial effects. To obtain figures of statistical value concerning the permanency of effects of irradiations upon fertility and to determine whether or not induced sterility was permanent, comparisons were made of the performance of only those flies carried through both the 9-day period and the 25-day period.

A word as to the meaning of the terms *sterility* and *fertility* as employed herein seems to be in order. Those experimental females, or females mated to experimental males, which deposited no eggs during the course of the laying period, or which produced eggs from which no adult flies emerged, are considered sterile. The term *fertility* applies only to those cases where a part, or all, of the eggs deposited developed into imagoes and is a measure of the number of eggs that succeeded in completing development.

Experimental Results

Sterility

The experiments reported in this paper provide data concerning the effects of irradiations upon sterility and fertility as defined above and throw some light upon the duration of these effects. Table I is a composite of the results upon the fertility of eggs deposited and the sterility produced. From this table it may be seen that five of the thirty-five control males were sterile, or 14.3 per cent. The same percentage of control females proved to be sterile. Of twelve males, treated as adults, two were sterile, or 16.66 per cent. Comparing this figure of sterility to that found for the control males we find a difference of 2.3 ± 8.09 per cent,—a difference too small to be significant. Treatment of larvæ at the ages of 72–84 hours rendered 33.33 per cent of the males sterile, while of males from larvæ treated at the ages of 24–36 hours, 50 per cent were found to be incapable of reproduction. The percentage of sterility found in the group of males derived from larvæ treated at 72–84 hours of age differs from that of the control group by 19.05 ± 8.29 per cent. Males obtained from larvæ treated at 24–36 hours of age differ in sterility from controls by 35.72 ± 6.7 per cent.

TABLE I

A Table Showing the Fertility of Flies 9–17 Days after X-ray Treatment: Dosage D5

Age at treatment	Number treated	Sex	Number fertile	Number sterile	Percentage sterile	Difference in percentage of sterility	Eggs laid	Flies	Average percentage of fertility	Difference in percentage of fertility
Adult	12	♂	10	2	16.66	2.3 ± 8.09	776	380	44 ± 5	45 ± 5.2
72–84 hr. larvae	15	♂	10	5	33.33	19.05 ± 8.29	1080	648	58 ± 5.2	28 ± 5.4
24–36 hr. larvae	20	♂	10	10	50	35.72 ± 6.7	923	502	52 ± 2.3	34 ± 2.8
Adult	15	♀	10	5	33.33	19.05 ± 9.1	915	641	70 ± 1.0	16 ± 1.8
72–84 hr. larvae	11	♀	10	1	9.0	−4.19 ± 7.8	1000	714	71 ± 3.4	15 ± 3.8
24–36 hr. larvae	17	♂	10	7	41.7	26.89 ± 8.3	933	756	81.3 ± 2.5	4.7 ± 2.5
Control	35	♀	30	5	14.3	—	2979	2571	86 ± 1.5	—

The sterility found in the various groups of females parallels that observed in males treated at corresponding ages. When females were treated as adults 33.33 per cent of the individuals were sterile. Subtracting the percentage of sterile controls from this figure leaves a difference of 19.05 ± 9.1 per cent. The group of females from larvæ irradiated at the ages of 72–84 hours contained very few sterile flies,—one in eleven, or 9.0 per cent. Here the percentage of sterile individuals was less than in the control group. However, it may be seen that a high degree of sterility maintained among females from larvæ treated at 24–36 hours of age. In this group 41.7 per cent of the individuals were

TABLE II

A Table of Calculations

Comparison of the fertility of treated and control flies

Percentage of fertility in controls	Percentage of fertility in treated	Difference in percentage	Difference / probable error
86 ± 1.5	Adult ♂ 44 ± 5.0	42 ± 5.2	8.07
86 ± 1.5	72–84 hr. ♂ 58 ± 5.2	28 ± 5.4	5.2
86 ± 1.5	24–36 hr. ♂ 52 ± 2.3	34 ± 2.8	12.1
86 ± 1.5	Adult ♀ 70 ± 1.0	16 ± 1.8	8.9
86 ± 1.5	72–84 hr. ♀ 71 ± 3.4	15 ± 3.8	3.94
86 ± 1.5	24–36 hr. ♀ 81.3 ± 2.5	4.7 ± 2.5	1.92

Comparison of fertility in sex of treated flies

Percentage of fertility	Percentage of fertility	Difference in percentage	Difference / probable error
Adult ♀ 70 ± 1.0	Adult ♂ 44 ± 5.0	26 ± 5.8	4.9
72–84 hr. ♀ 71 ± 3.4	72–84 hr. ♂ 58 ± 5.2	13 ± 6.2	2.1
24–36 hr. ♀ 81.3 ± 2.5	24–36 hr. ♂ 52 ± 2.3	29.3 ± 3.4	8.6

sterilized. The sterility in this case differs from that found in control females by 26.89 ± 8.3 per cent, the difference being 3.24 times the probable error.

Fertility of Eggs Deposited

The table of calculations (Table II) was devised to compare the fertility of treated groups to the fertility displayed by the control series. From the data on control flies we find the mean fertility of the group to be 86 ± 1.5 per cent. When adult males were treated with irradiations, the mean fertility of eggs subjected to fertilization by them was 44 per cent with a probable error of ± 5 per cent. This group of treated

males differs in fertility from the controls by 42 ± 5.2 per cent, the difference being 8.7 times its probable error. Males treated in larval stages exhibit no such marked reduction in ability to fertilize eggs as do males irradiated after metamorphosis. The fertility of eggs collected from matings of males X-rayed at 72–84 hours of age amounts to 58 per cent of all eggs secured from the group, the probable error being ± 5.2 per cent. The mean fertility of this group is found to differ from that of the controls by 28 ± 5.4 per cent; a difference 5.2 times the probable error. The fertility of males treated at the larval ages of 24–36 hours is slightly lower than that of males irradiated when 72–84 hours old. Of 923 eggs collected from females crossed to males that were X-rayed at 24–36 hours of age, 52 ± 2.3 per cent produced adults. This figure (52 ± 2.3 per cent) subtracted from the fertility displayed by controls (86 ± 1.5 per cent) leaves the significant difference of 34 ± 2.8 per cent.

Thus far the fertility of treated males only has been considered. A review of the data obtained from treated females, Table II, shows that eggs of females, irradiated thirty hours after emerging from the pupal stage, exhibit a mean fertility of 70 ± 1.0 per cent. A comparison of the egg fertility of this group to that of the control group reveals that, due to the effects of irradiation, 16 ± 1.8 per cent of the eggs from females treated as adults failed to complete their development. This figure, 8.9 times its probable error, is highly significant. The mean fertility of eggs from females treated as 72–84 hour larvæ was 71 ± 3.4 per cent, or 15 ± 3.8 per cent less than that of controls. However, treatment of 24–36 hour larvæ reduced the fertility of eggs deposited by females from such larvæ by only 4.7 ± 2.5 per cent. Since this figure is only 1.92 times its probable error no significance can be attached to it.

Permanence of Effects upon Fertility and Sterility

The volume of data on the permanence of the effects of irradiation upon fertility is such as to exclude it from this publication and a brief review of the findings is included in the section on discussion.

From a study of the data contained in Table III it may be seen that in some cases flies which were fertile at 9 days after treatment became sterile within 25 days after irradiation. Nine such cases appear among 52 treated individuals. Of 14 flies found sterile at 9 days after treatment, 3 regained their fertility within 25 days, or 21.4 per cent of the flies were temporarily sterilized by irradiations. On the other hand, 6.66 per cent of the control flies found fertile 9 days after mating were sterile in the 25-day period, while none of the controls that were sterile

TABLE III

A Table Comparing the Sterile Flies of Two Periods

Serial Number	9–17 days after treatment			25–33 days after treatment		
	Eggs	Flies	Sterile	Eggs	Flies	Sterile
A ♂ 6	83	2	—	100	0	1
A ♂ 9	7	3	—	37	0	1
A ♀ 6	100	39	—	56	0	1
A ♀ 13	0	0	1	0	0	1
A ♀ 14	0	0	1	7	0	1
A ♀ 15	0	0	1	21	0	1
C ♂ 10	59	10	—	0	0	1
C ♂ 11	0	0	1	100	89	—
C ♂ 13	45	0	1	6	0	1
C ♂ 14	0	0	1	100	55	—
C ♂ 15	0	0	1	6	0	1
C ♀ 2	100	82	—	24	0	1
B ♂ 4	55	1	—	9	0	1
B ♂ 7	100	88	—	36	0	1
B ♂ 10	100	2	—	3	0	1
B ♂ 15	0	0	1	24	0	1
B ♂ 16	0	0	1	0	0	1
B ♂ 18	0	0	1	0	0	1
B ♂ 19	29	0	1	38	0	1
B ♀ 8	50	42	—	1	0	1
B ♀ 12	6	0	1	27	0	1
B ♀ 14	36	0	1	0	0	1
B ♀ 17	18	0	1	11	3	—
Total	788	269	14	606	147	20
Controls						
D 24	79	33	—	38	0	1
D 31	100	0	1	100	0	1
D 32	100	0	1	100	0	1
D 33	100	0	1	100	0	1
Total	379	33	3	338	0	4

9 days after mating produced offspring during the 25-day period of mating.

DISCUSSION

Little more can be said by way of discussing the sterility effects of irradiation than to point out the percentage differences between the treated and control groups. It is unnecessary to apply a mathematical formula to determine that there is no difference in the sterility of males

treated as adults and that of the control males. A larger percentage of sterility is found if males derived from larvæ irradiated at 72–84 hours of age are tested. In this group the percentage of sterile individuals differs from that of the controls by 19.05 ± 8.29 per cent, a figure not mathematically significant but perhaps suggestive. However, males obtained from larvæ treated at 24–36 hours of age differ in sterility from controls by 35.72 ± 6.7 per cent. In this case there is no doubt that X-rays produced sterile individuals, since the difference is 5.33 times the probable error.

Analysis of the data on females shows the sterility of only one group to differ significantly from the sterility found for the control females. The percentage of sterile flies found among females from larva X-rayed at 24–36 hours of age differs from that of the control females by 26.89 ± 8.3 per cent. Therefore, in the case of either males or females taken from larvæ treated at 24–36 hours of age, irradiation produces sterile individuals. It is also quite probable that X-rays produced sterility in the other treated groups tested, but the dosage used was too light and the numbers were too small for obtaining definite proof.

In normal stocks of *Drosophila* the proportion of eggs which hatch at room temperature is remarkably constant and the percentage of fertile eggs deposited is extremely high. Packard (1927) reported an average fertility of 97 per cent, basing his report upon the number of larvæ appearing in the cultures. The thirty fertile controls used in this experiment deposited 2979 eggs, 86 per cent of which completed metamorphosis. When we consider that Packard used a different strain of flies and based the fertility upon larvæ rather than adults that emerged, this figure compares very favorably with the fertility which he observed.

Table II compares the fertility of the treated groups to the fertility displayed by the control series. When the mean fertilities of the different groups of treated males are compared to the fertility of eggs obtained from controls, we find the differences ranging from 42 ± 5.2 per cent, if males treated as adults are compared, to 28 ± 5.4 per cent, if male larvæ irradiated at 72–84 hours of age are used. It appears at once that the reductions of fertility effected by irradiations are significant. Clearly, the action of X-rays upon the germ cells of the male rendered a large proportion of those cells incapable of entering into the fertilization process, or produced effects within the sperm cells which inhibited the development of the zygotes of which such cells have become a part.

From the experimental data it is obvious that the fertility of males as defined in this paper is reduced by the action of X-rays irrespective of the developmental stage irradiated. The highest degree of fertility

displayed by any of the three groups of males tested represents a reduction in fertility of 28 ± 5.4 per cent, while the greatest reduction of 42 ± 5.2 per cent is evidence of the extreme effects of X-rays upon the fertility of males treated during adult life. These statements are, however, to be regarded, not as facts which must recur since the difference between any two groups of treated males (the greatest difference being 14 ± 7.2 per cent, or 1.94 times the probable error) is so small as to be insignificant. In case these suggestions point in the right direction, as the data indicate, it is well to note that only the fertility of eggs is considered, sterile flies being eliminated. This might be called the "net" fertility. If we consider the sterile flies produced in the various groups plus the egg fertility, opposite results are obtained. That is to say, the "gross" fertility of adult males is affected less than that of males treated in the larval stages.

Going briefly into the findings of the fertility of irradiated females, we see that the fertilities of only those females from larvæ treated at 72–84 hours of age and the females irradiated as adults differ significantly from that of the control group. Treatment of 24 to 36-hour larvæ reduced the fertility of eggs deposited by females from such larvæ by only 4.7 ± 2.5 per cent. Since this figure is only 1.92 times its probable error, we cannot conclude that irradiation reduces the fertility of eggs if the females are treated at the larval age of 24–36 hours. This result may be due to the severe action of X-rays, which produces sterile individuals in a large number of the cases in which the gonads are affected, or to an interruption of developmental processes which inhibits the appearance of the individual whose gonads are affected by the absorption of radiant energy. Be that as it may, the data show clearly that the fertility of females treated in the later stages of development is reduced by the action of irradiations.

It is interesting to note that there is no significant difference in the egg fertility observed if the different groups of treated males are compared, or if the three groups of irradiated females are compared with one another. On the other hand, if we compare the fertility of males to the fertility of females treated in the same stage of development, we find in each case, with one exception, that the fertility of eggs is more affected by treating males than by treating females. The fertility of treated adult males is 26 ± 5.8 per cent lower than that of treated adult females. This figure is 4.5 times the probable error and without doubt is significant of an inherent difference in the germ cells of the sexes which accounts for the greater susceptibility of the sperm cells to irradiations. The males and females irradiated at the larval age of 72–84

hours show a difference in fertility of 13 ± 6.2 per cent, favoring the females, or a figure 2.1 times its probable error, which is slightly indicative of a greater reduction in the fertility of males than in the fertility of females. The fertility of the males derived from larvæ treated earlier in life (24–36 hours) differs greatly from that of the females. In this case the fertility of the males is 29 ± 3.4 per cent lower than the fertility of females.

Summarizing the effects of X-rays upon the fertility of flies treated at different stages of development we may say, if sterile flies are eliminated, that (1) except by treatment of female larvæ 24–36 hours of age, the fertility of either sex may be reduced by X-ray treatment irrespective of the age at which the flies are irradiated, (2) the effects of irradiation upon the fertility of female *Drosophila* are at a minimum when 24 to 36-hour larvæ are treated, (3) there is no significant difference in the fertility of a sex, regardless of the age at which the flies are treated, but (4) it is evident that if treatment is administered to opposite sexes of *Drosophila* in the same stages of development, X-rays of the dosage used affect the fertility of males to a greater degree than that of females, irrespective of the age at which the flies are subjected to treatment.

It is curious to note that a dosage of X-rays considerably higher than that here used had been found by Muller to have the opposite effect,—reducing the fertility more in adult females than in adult males.

Two questions arise: Are the effects which irradiations produce upon fertility of a permanent nature? Is sterility, once induced, to remain a characteristic of the individual throughout life, or is such sterility of a temporary nature? To determine the answers to these important and interesting questions two periods of egg collection were observed. These were fully described above as the 9 to 17-day period and the 25 to 33-day period and will be referred to as such in the following discussion.

Comparisons of average fertilities found for the groups during the 9 to 17-day period with those found for the 25 to 33-day period appear at first glance to indicate that eggs deposited in the second period exhibited a higher degree of fertility than eggs deposited in the first period. Considering the number of flies included in these calculations it is hardly advisable to depend upon a comparison of the fertilities exhibited by the groups during the two periods. The numbers are so small and the variations in the fertility of the individual flies so great that the difference in fertility exhibited by one or two flies may influence the average percentage of the group in such manner as to make it slightly higher for the 25 to 33-day period, whereas had these few flies varied as the

remainder of the group the average would have been lower for the 25 to 33-day period than for the 9 to 17-day test. But if the fertilities exhibited by the individual flies at nine days after irradiation are compared to those exhibited twenty-five days after treatment, it becomes evident that some of the flies were more fertile in the second than in the first period, while others were less fertile in the second period than in the first. In either sex, irrespective of the stage in development at which the flies were treated, the fertility displayed twenty-five days after irradiation was often different from that exhibited nine days after the administration of X-rays. Although it is certain that the fertility of untreated flies decreases with age, we cannot say whether the fertility of irradiated individuals will increase or decrease with the passage of time. We can, however, say with some degree of certainty that the percentage of fertile eggs deposited, or fertilized by treated flies, varies greatly at different dates after irradiation. After X-ray treatment the rate of fertilization is highly variable irrespective of the sex which has been subjected to irradiations. In other words, a large number of eggs deposited by treated flies during one period may be viable, while of those collected at some other date relatively few may be capable of development.

From a close study of the data included in Table III we find a few instances in which flies were fertile at nine days after treatment but became sterile within twenty-five days after irradiation. Of the fertile treated flies 17.3 per cent became sterile between the seventeenth day and the twenty-fourth day following X-ray treatment. Of fourteen flies sterile at nine days after treatment, three regained their fertility within twenty-five days, 21.4 per cent of the sterile flies were only temporarily so. There is, perhaps, no significant difference in the percentage of flies regaining fertility and the percentage becoming sterile, but significance may be attached to the fact that some flies sterilized by X-rays were found to become fertile with the passage of time following irradiation while none of the four flies found sterile among the controls ever became fertile. On the other hand, 6.66 per cent of the control flies found fertile nine days after mating were sterile in the 25-day period, indicating a natural tendency for normal flies to become sterile with age.

From the second period of egg collection (25–33 days after treatment of flies, or after mating controls) we learn that (1) the percentage of fertile eggs deposited at nine days and twenty-five days after irradiation is not constant, (2) flies may be rendered temporarily sterile by X-ray treatment, but sterile untreated flies fail to become fertile with time and (3) untreated flies decrease in fertility with age.

It seems reasonable to conclude that the effects of irradiation upon the fertility of *Drosophila melanogaster* are not permanent, and that fertilization may be effected rather irregularly by treated flies. Certainly, since flies are temporarily sterilized by X-rays, the action of irradiations in producing sterile individuals is not always permanent.

The author wishes to express thanks to Professor J. T. Patterson and to Professor H. J. Muller for their many helpful suggestions during the course of the experiments.

Conclusions

1. With the moderate dosage used, the degree of fertility exhibited by *Drosophila* eggs is more affected by the treatment of males than by the irradiation of females of the same age, regardless of the stage in development at which irradiations are administered. Females from cultures of larvæ treated at 24–36 hours of age display, when fertile at all, a higher degree of fertility than flies of either sex, irrespective of the age at which they are irradiated.

2. Irradiation of larvæ 72–84 hours of age sterilizes a smaller proportion of the flies of either sex than treatment of any other larval stage, and less sterility is found among females when larvæ are treated at this age than in any other group of flies tested.

3. When larvæ only are irradiated more males are sterilized than females.

4. The effects of irradiation upon fertility are not permanent. Irradiated flies are variable in the degree of fertility which they exhibit at different dates after treatment. Fertility may increase with the passage of time following treatment, but the fertility of control flies decreases with age.

5. The action of X-rays in producing sterile flies may often have only a temporary effect.

Literature Cited

Hanson, F. B., 1928. The Effects of X-rays on Productivity and the Sex Ratio in Drosophila melanogaster. *Am. Nat.*, **62**: 352.

Hanson, F. B., and Francis R. Ferris, 1929. A Quantitative Study of Fecundity in Drosophila melanogaster. *Jour. Exper. Zool.*, **54**: 485.

Mavor, J. W., 1927. A Comparison of the Susceptibility to X-rays of Drosophila melanogaster at Various Stages of Its Life-Cycle. *Jour. Exper. Zool.*, **47**: 63.

Muller, H. J., 1928. The Problem of Genic Modification. *Zeitschr. f. ind. Abst. u. Vererb. Sup.*, **1**: 234.

Packard, Charles, 1927. The Quantitative Biological Effects of X-rays of Different Wave-Lengths. *Jour. Cancer Res.*, **11**: 1.

Packard, Charles, 1928. A Comparison of the Quantitative Biological Effects of Gamma and X-rays. *Jour. Cancer Res.*, **12**: 60.

THE EFFECT OF TEMPERATURE ON THE LEG POSTURE AND SPEED OF CREEPING IN THE ANT LASIUS

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INTRODUCTION

The present paper is an attempt to investigate the influence of temperature upon the locomotion of the ants, *Lasius niger* and *L. umbratus*. In particular it deals with the correlation between temperature and leg-spread, and temperature and speed.

Shapley has determined the correlation between temperature and speed of progression for several species of ants. His first paper (Shapley, 1920) reported that, for *Liometopum apiculatum*, "... an empirical curve is obtained that for any temperature throughout a range of 30° centigrade gives the speed with an average probable error of 5 per cent for one observation. Conversely, from a single observation of the ant-speed, the temperature can be predicted within 1 degree centigrade."

In his second paper (1924), Shapley applied the Van't Hoff-Arrhenius equation to his data. He stated: "A constant value of μ at about 11,000, for temperatures from 20° C. to 35° C., appears to be justified. But further observation is necessary to test the indicated variability of μ for temperatures below 20° C." Assuming a break at 20° C., he approximates μ as 20,000 for the lower temperatures.

Hoagland (1931) has studied the influence of temperature on learning in ants, but this does not concern us here.

Wheeler's book (1913) may be consulted for an account of the ecological aspect of the influence of temperatures on ants. Fielde (1905), in particular, has observed the effect of temperature on the development of ants.

LEG-SPREAD AND TEMPERATURE

The relationship between leg-spread and temperature was determined as follows: Ants were permitted to crawl over smoked kymograph paper, lying in a covered Petri dish. The dish was placed in either a dark, constant-temperature chamber, or in a glass vessel immersed in the water of a thermostat. In the latter case, light was eliminated by the addition of India ink to the water.

The records were then shellacked, after which microphotographs were made, with the helpful advice of Professor A. Petrunkevitch.

The trails made by the metathoracic legs are easily seen and studied on the microphotographs (Fig. 1). Specimens of *L. umbratus,* taken from a genetically homogeneous colony, were utilized. In all cases it

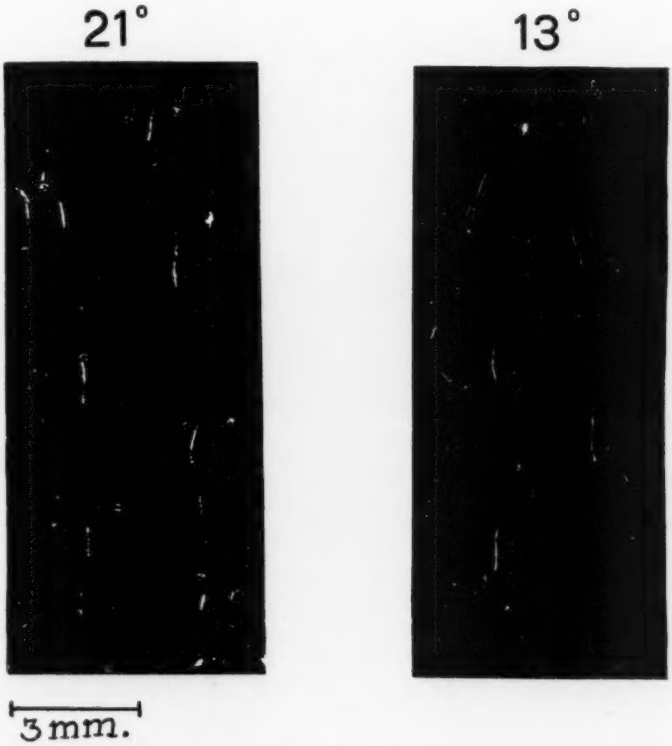

FIG. 1. Microphotographs of the footprints of *Lasius umbratus* (No. 124) on smoked kymograph paper. These records were taken in the dark at 21° and at 13° C. after one-half hour for thermal adjustment. The greater leg-spread at 21° is evident but is more striking over a wider temperature difference. The long streaks are made by the hind legs. These photographs are not re-touched.

was found that as the temperature rose, the legs were held further apart. Since the increase in leg-spread per degree rise in temperature is very small, the phenomenon is best illustrated over a fairly large temperature difference.

A typical instance of this phenomenon is demonstrated by Specimen 124 (Figs. 1 and 2). At 12° C. the leg-spread was 2.36 ± .31 mm.; at 21° C., 2.81 ± .20 mm.; at 25° C., 2.88 ± .23 mm.

Speed and Temperature

Methods

The thermostat consisted of a large glass jar filled with water. The temperature was regulated by a mercury toluene contact and by the addition of ice or hot water. Bubbles of compressed air thoroughly stirred the system.

The ants crawled in a glass tube 15 cm. long, of 5 mm. internal diameter, on which lines were etched at 5 cm. intervals. The ends of

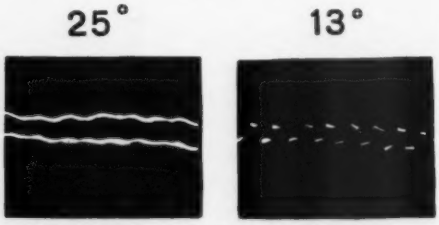

Fig. 2. Trails made by *Lasius umbratus* crawling over smoked kymograph paper; the actual tracings are covered with white paint to demonstrate the width of the paths. Upper tracing ant No. 124, 25° C. Lower tracing, same individual at 13°. At the higher temperature the hind legs are further apart and with many specimens make an unbroken tracing: see text.

this tube were lightly plugged with moist cotton and were attached by rubber tubing to spirals of glass tubing, the free ends of which extended out of the thermostat. Thus air adjusted to a given temperature could circulate through the system. This we think necessary since ants died when left overnight if the 15 cm. glass tube was corked at both ends, but survived if it was lightly plugged with cotton.

A thermometer placed in the 15 cm. glass tube indicated that about ten minutes was necessary for the air within the tube to reach the same temperature as the surrounding water.

Actively crawling ants were taken from the two genetically homogeneous formicaries of *L. umbratus* and *L. niger,* placed in the 15 cm. glass tube, and, after an interval of at least ten minutes, were clocked with a stop watch as they crawled between the 5 cm. lines. When necessary the tube was gently tapped to induce crawling. Actively crawling specimens were used because observations over a long period indicate a rhythm of activity and rest in the colony as a whole.

A diffuse light of approximately 25-foot candles flooded the entire thermostat. We do not regard the light conditions as a disturbing factor.

Data

The data accumulated for *L. umbratus* are graphed in Fig. 3. The ordinates represent the log of the rate of progression:

$$\frac{1}{\text{time in seconds for 5 cm.}} \times 100.$$

The abscissæ are the reciprocals of the absolute temperatures. These units were used so that the data could be applied to the Van't Hoff-Arrhenius equation.

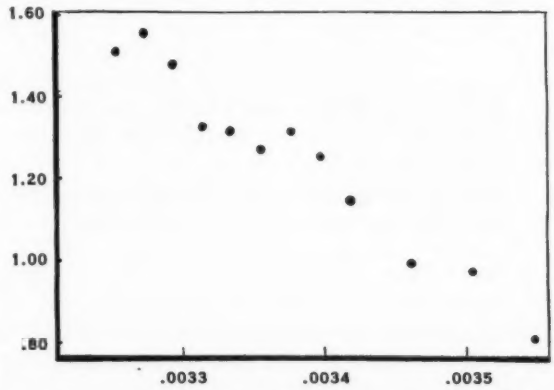

FIG. 3. Rate of creeping as controlled by temperature in *Lasius umbratus*. The log of the progression rate, $\frac{1}{\text{time in secs. for 5 cm.}} \times 100$, is plotted against the reciprocal of the absolute temperature (abscissæ). Each point is the average of 8–20 stop watch readings. Because of the scattering of points, no certain line could be drawn.

Each point on the graph represents from 8 to 20 readings. The temperature range is limited by the facts that below 10° C. it is very difficult to induce crawling, while above 30° C. the specimens are injured, some dying at the latter temperature.

The graph clearly shows that the speed of progression is influenced by temperature. On the basis of centimeters per second, the increase is from .3 cm. per second (average) at 10° C., to 1.6 cm. per second at 24° C. Sample time records for 5 cm. runs are shown in Table I.

The value of μ, the energy of activation of the catalyst, in the Van't Hoff-Arrhenius equation, was called the temperature characteristic by

TABLE I

Rate of locomotion. Mean time, based on 8 trials, ant crawling 5 centimeters in each.

Temperature	Individual	Time
° *C.*		*seconds*
12	120 (umbratus)	11.6
18	114 (umbratus)	4.3
16	97 (niger)	7.1
20	97 (niger)	5.5
25	99 (niger)	2.8

Crozier (1924), when he applied the equation to biological phenomena. The form of the equation we used was:

$$\ln \frac{R^2}{R^1} = \frac{\mu}{2}\left(\frac{1}{T^1} - \frac{1}{T^2}\right)$$

R^1 is the rate at T^1, and R^2 is the rate at T^2. T is the absolute temperature. In using the equation a line is drawn through the graphed points, and the rates are then calculated from it.

In Fig. 3 it will be noticed that there is a greater scatter of points in the higher range. Furthermore, since the lower range also exhibits scattering, we do not put too much faith in the rectilinear relation. However, we have calculated a value of μ for it, which is 22,500 calories.

The data accumulated for *L. niger* are graphed in Fig. 4. The linear relationship between temperature and speed is evident, and a sharp break at 20° C. is easily seen. From 15° to 20° C. μ has a value of 10,700

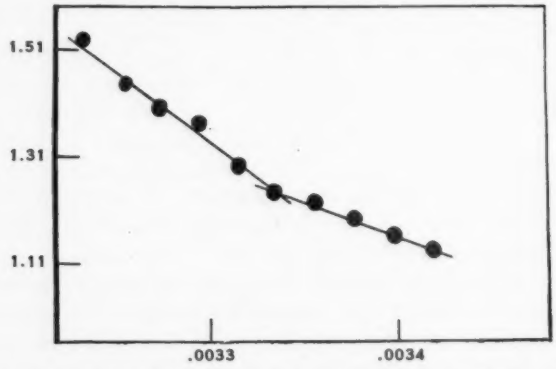

FIG 4. Rate of creeping as controlled by temperature in *Lasius niger*. Abscissæ and ordinates on same basis as in Fig. 3. Note the clear-cut break at 20° C., the critical temperature.

TABLE II

Values of μ (Temperature Characteristic Determined)

Species	Activity	Temperatures °C.	μ
L. umbratus	Speed of progression	10–19	22,500
L. umbratus	Frequency of leg movements	12–25	12,700
L. niger	Speed of progression	16–20	10,700
L. niger	Speed of progression	20–25	22,900

calories; from 20° to 25° C., a value of 22,900 calories. Sample time records are shown in Table I.

The foregoing values of μ are based on speed, which itself must be dependent in part on the frequency of leg movement—a difficult quantity to determine. Several attempts to do this, however, were made. The method was simply to observe the ant in the thermostat and clock the time for ten steps of the metathoracic leg. At low temperatures this is not difficult, but in the higher range the frequency determined cannot be very accurate. *L. umbratus,* Specimen No. 128, showed the following mean frequencies per second, based on 7 readings for each temperature: 12° C., 2.13; 18° C., 3.45; 25° C., 5.55. This gives μ the value of 12,700 calories.

DISCUSSION

In the case of *L. umbratus* we have experienced difficulty in fitting a curve to the experimental data for speed of progression, so that we might utilize the Van't Hoff-Arrhenius equation. Our data plainly show that speed of progression increases as the temperature rises, as do leg-spread and frequency of leg movement. Furthermore, leg-spread and frequency of leg movement no doubt largely determine speed of progression. But each of these three has its own rate of increase. We desire to point out that the locomotion of ants involves a number of complex factors.

Crozier and Stier (1925) have shown that in the tent caterpillar speed of progression is not a simple exponential function of $1/T_{abs}$, but that frequency of abdominal peristaltic locomotor waves, a factor of the former, is, giving the value of 12,200 calories.

Crozier (1925) pointed out that the value for μ of 12,200 calories in arthropods has been found for various rhythmic types of neuro-muscular activity (respiratory excepted). These include speed of progression of a diplopod, the calls of the katydid and cricket, and the flashing of the firefly. Also, our colleague Professor G. Evelyn Hutchinson (1928)

has found a μ of 11,500 for the speed of progression of an interesting South African isopod, *Preatoicus capensis*.

Crozier's μ's for Shapley's 1920 *Liometopum* data were 25,900 for below 16° C., and 12,200 for from 16° to 38° C. Our findings roughly approximate these, except in the case of *L. umbratus*, as can be seen in Table II. It should be remembered that the data for *speed* of progression in *L. umbratus* are none too good a basis for the calculation of μ.

Summary

1. In the progression of *L. umbratus* the leg-spread and the frequency of leg movements increase as the temperature rises.

2. The speed of progression as influenced by temperature in *L. niger* shows a critical temperature at 20° C., and yields two values for μ: 10,700 between 16–20° C., and 22,900 between 20–25° C.

LITERATURE CITED

Crozier, W. J., 1924. Critical Thermal Increment for the Locomotion of a Diplopod. *Jour. Gen. Physiol.*, **7**: 123.

Crozier, W. J., and T. B. Stier, 1925. Temperature Characteristic for Locomotor Activity in Tent Caterpillars. *Jour. Gen. Physiol.*, **9**: 49.

Fielde, A. M., 1905. Temperature as a Factor in the Development of Ants. *Biol. Bull.*, **9**: 361.

Hoagland, H., 1931. A Study of the Physiology of Learning in Ants. *Jour. Gen. Psychol.*, **5**: 21.

Hutchinson, G. E., 1928. On the Temperature Characteristics of Two Biological Processes. *South African Jour. Sci.*, **25**: 338.

Shapley, H., 1920. Thermokinetics of Liometopum apiculatum Mayr. *Proc. Nat. Acad. Sci.*, **6**: 204.

Shapley, H., 1924. Note on the Thermokinetics of Dolichoderine Ants. *Proc. Nat. Acad. Sci.*, **10**: 436.

Wheeler, W. M., 1913. Ants. New York: Columbia University Press.

OSMOTIC PROPERTIES OF THE ERYTHROCYTE

IV. Is the Permeability of the Erythrocyte to Water Decreased by Narcotics?

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I

In the course of studies connected with his well-known " Haftdruck " theory, Traube (1908) made the observation that certain lipoid-soluble substances, such as amyl alcohol, which are also known to possess narcotic properties, may, under appropriate conditions, exert an antihemolytic effect. This effect he interpreted as being due to " eine Verdickung der Lipoidschicht welche die Stabilität der Blutkörperchen gegen andere Hämolytika ändern muss." A few years later Arrhenius and Bubanović (1913) obtained similar results with chloroform, ethyl ether, ethyl and amyl alcohols, and benzene. As an example of these results one typical experiment with a hypotonic solution containing chloroform may be cited. In this experiment it was found after one hour at 37° C., followed by several more in the ice-box, that the percentage of hemolysis decreased from 60 per cent in the absence of chloroform to a minimum of 37 per cent in the presence of 0.2 per cent, rising again at higher concentrations. Arrhenius and Bubanović did not hesitate to conclude that " diese Wirkung beruht vermutlich auf einer Verlangsamung des Eindringens von Wasser in die Zellen." Among the more recent workers in this field, Yoshitomi (1920) found after exposures of 2 to 18 hours to hypotonic solutions, a lesser degree of hemolysis in the presence of certain concentrations of ether, chloroform, chloretone and amylene hydrate than in their absence. He suggested that this effect might be due either to a less ready entrance of water into, or a more ready escape of salts from, the cells. Jarisch (1921) also obtained an inhibition of osmotic hemolysis with alcohol, ether, amylene hydrate and urethane. While he did not state in very precise terms his conception of the antihemolytic action of these substances, his use in this connection of the term " wasserhemmend " would seem to indicate that his views were not unlike those of Arrhenius and Bubanović.

Since these and similar results obtained with the erythrocyte have frequently been cited in support of the view that narcosis is associated with a decreased cell permeability, it seems necessary for us to point out that such evidence is entirely inconclusive. In the first place, osmotic hemolysis is a complicated process involving not only (*a*) the entrance of water into the erythrocyte, but (*b*) the escape of hemoglobin, (*c*) the possible loss from the cell of salts and other osmotically active substances (see in this connection Ponder and Saslow, 1931) as well as (*d*) a variety of possible changes of diverse nature, not directly associated with permeability, in what is commonly and rather loosely called the "osmotic resistance" of the cell. While it is undoubtedly true that anything that decreases the permeability of the cell to water (factor *a*) will, in general, tend to delay osmotic hemolysis, the reverse statement is by no means true. A delay might equally well be produced by the operation of any or all of factors *b*, *c*, and *d*.

A more fundamental objection, however, to evidence of the type mentioned above, is that it is very unlikely that the investigators in question obtained from their experiments any real information as to the *rate* of hemolysis, which is the thing of greatest importance in connection with questions of permeability to water. It is, of course, conceivable that if at the end of some single arbitrarily selected time a lesser degree of hemolysis is obtained in the presence than in the absence of a narcotic, the difference might be due to a slower rate of progress in the former case towards the same final end state of the system. On the other hand, the possibility must be considered that the narcotic exerts its effect primarily on the degree of hemolysis ultimately attained; in that case, with no knowledge of the position of equilibrium towards which the system is proceeding—or which it may indeed have reached at the time the observation is made—no valid conclusions whatever can be drawn as to the fundamental rate of the hemolytic process.

Though this principle would seem to be a self-evident one, it has been very frequently disregarded in the past, not merely in studies on hemolysis, but in other fields of physiological work as well. It should be most strongly emphasized, therefore, that while it is possible to study a position of equilibrium with no exact knowledge of the rate at which it is attained, the reverse procedure of attempting to draw conclusions concerning a rate, with no information whatever as to the end state which the given system is approaching, is entirely unwarranted and can lead only to confusion (see in this connection Jacobs, 1928). In the present paper the distinction between the effects of certain urethanes on the "equilibrium" and the "rate" factors concerned in osmotic hemolysis will be illustrated; and it will further be pointed out that since

as far as we are aware the work of previous investigators in this field makes no such distinction, it can be expected to throw no real light on the question of the permeability of the erythrocyte to water.

II

As a first step in the separation of the two types of factors, it seemed important to obtain curves representing the entire course of hemolysis from its beginning until further change had ceased. This necessary type of information, which, as has been mentioned, has apparently not been supplied by previous workers, is particularly easy to obtain by the method of one of the authors (Jacobs, 1930). A typical experiment is represented in Fig. 1 in which the erythrocytes were those of the ox and the narcotic was ethyl urethane. The blood in this, as in all the

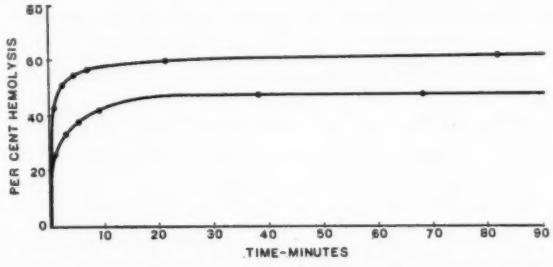

FIG. 1. Course of hemolysis of ox blood in buffered 0.090 M NaCl in the presence (lower curve) and absence (upper curve) of 0.3 M ethyl urethane: pH, 7.4; temperature, 20° C.

other experiments here described, was thoroughly and almost instantaneously mixed with the solutions used in the proportion of approximately 1 to 500. Kymograph records were made of the ensuing hemolysis and from these the curves in the figure were reconstructed. Because of the enormous importance of pH and temperature changes in experiments involving equilibria, the precautions described by Jacobs and Parpart (1931) were employed in all such experiments, the pH being kept almost constant in this case at approximately 7.4, and the temperature at 20° C.

An inspection of Fig. 1 shows very clearly that under the conditions of this experiment the effect of ethyl urethane upon the final end-point reached by the system is far more striking than any possible effect it might have upon the rate of hemolysis as such. Thus, in the absence of the urethane, a degree of hemolysis of approximately 61 per cent was attained in between 20 and 30 minutes, and after that time no further

change occurred; in its presence a final degree of hemolysis of 48 per cent was reached in about the same time, and this likewise underwent no further change. Very similar results were obtained in a number of other experiments. It is evident, therefore, that no information about the rate of hemolysis can be secured in such cases by observing at the end of some arbitrarily selected time the mere degree of hemolysis that then happens to exist.

It is to be noted that the antihemolytic effect of ethyl urethane is not a simple osmotic one due to the greater total concentration of the solution containing it. Such an effect could be obtained only with a non-penetrating substance, since otherwise the solute molecules would distribute themselves inside and outside the cell in such a way that their osmotic effects would everywhere balance. In the case of the urethanes, however, not only is the penetrating power for cells in general known to be extremely high, but a number of hematokrit measurements made in the course of these experiments showed the absence of any measurable osmotic effects on cell volume. As a matter of fact, though the concentration of the ethyl urethane in this particular experiment was 0.3 M, the antihemolytic effect was only that which would have been produced osmotically by an increase in concentration of possibly 0.006 M in a solution of a non-penetrating non-electrolyte.

As to the effect of ethyl urethane on the fundamental rate of hemolysis, apart from that on the final degree attained, this cannot be determined by a mere inspection of such curves as those in Fig. 1. Even though a measurably longer time were required to attain a given degree of hemolysis in the presence than in the absence of the narcotic, it would be impossible to be certain, without a fairly complicated mathematical analysis of the results, how much of the observed effect was due merely to the shift in the final equilibrium. Since, therefore, even under the relatively favorable conditions provided by the possession of two complete hemolysis curves it is very difficult to draw conclusions about the effect of a narcotic on the rate of the process, what information of value could conceivably be obtained from the knowledge of only a single point on each curve? As a matter of fact, most of the observations mentioned in the introductory paragraph were taken at times long after those at which the final equilibrium must have been attained and could, therefore, by no possibility throw any light upon the rate of hemolysis, and by implication, upon the possible rate of entrance of water into the cells. Though, as will be shown later, the conclusion of previous investigators that the rate of entrance of water into the erythrocyte is slowed by the presence of a narcotic may in itself be entirely correct, it may not validly be drawn from the data they have presented.

Having found in experiments of the type of that represented in Fig. 1 that the final equilibrium condition is usually reached within 20 or 30 minutes, and in any case in less than an hour, it seemed desirable to study by more quantitative methods than those previously used the effects of varying the concentration of the narcotic. In Table I are given the results of one such experiment with ethyl urethane in which the percentages of hemolysis reached in one hour in the presence of different concentrations of this substance were determined. The concentration of NaCl used was selected as a favorable one for the particular sample of ox blood employed. The pH in this case was approximately 7.35 and the temperature, as before, 20° C. It will be noted that as the concentration of urethane increased, the degree of hemolysis decreased until a maximum effect was reached probably somewhere between

TABLE I

Degree of hemolysis attained by ox erythrocytes in one hour in a buffered 0.085 M NaCl solution containing different concentrations of ethyl urethane.
pH, 7.35; temperature, 20° C.

Concentration of urethane	Percentage of hemolysis
—	75
0.0078	75
0.0156	74
0.0313	74
0.0625	70
0.125	70
0.25	68
0.5	73
1.0	100

0.25 M and 0.5 M. Beyond this point a hemolytic effect of the narcotic became evident. The general results of this entirely typical experiment with ethyl urethane do not differ in principle from those obtained by Arrhenius and Bubanović with chloroform, if it be assumed, as was almost certainly the case, that what these investigators measured was the final position of equilibrium of the system.

In another experiment, whose results are given in Table II, the general procedure was reversed by keeping the concentration of urethane constant at 0.1 M and varying that of the hypotonic solution. In this way the antihemolytic effect of the narcotic on a considerable proportion of all the erythrocytes in the blood could be observed. It will be noted that although the results show certain minor irregularities, there is in no case any departure from the previously observed antihemolytic effect. Similar, though less complete, results were also obtained with several other urethanes, as well as with ethyl alcohol. Taking together these results and those reported by previous workers, the evidence

seems to be entirely consistent that the degree of hemolysis ultimately attained in a hypotonic solution may be reduced by a variety of narcotic substances in proper concentrations. This fact, however, though of interest in other ways, throws little or no light upon the question of the effect of narcotics upon the permeability of the erythrocyte to water. What is needed is information not about the final equilibrium but about the fundamental rate at which hemolysis occurs.

III

It has been pointed out in another place by one of the authors (Jacobs, 1928) that in cases where the position of equilibrium of a

TABLE II

Degree of hemolysis attained by ox erythrocytes in one hour in buffered NaCl solutions of different concentrations in the absence and presence of 0.1 M ethyl urethane.
pH, approximately 7.35; temperature, 20° C.

Concentration of NaCl	Percentage of Hemolysis	
	Urethane absent	Urethane present
0.098	82	78
0.099	78	74
0.100	73	69
0.101	71	65
0.102	69	59
0.103	67	55
0.104	63	53
0.105	57	48
0.106	53	44
0.107	50	37
0.108	45	38
0.109	41	33
0.110	34	22

hemolytic system is influenced by the same factor whose effect on the rate of hemolysis it is desired to study, the general rule should be followed of keeping the system at all times as far away from equilibrium conditions as possible. Thus, in investigating the effect of temperature upon the rate of osmotic hemolysis, it was found that where only water or very strongly hypotonic solutions were employed consistent and plausible results could be obtained, while with less strongly hypotonic solutions the results were erratic and at first sight inexplicable. Similarly, in the case of narcotics, which have been shown in the preceding section to affect in a striking manner the degree of hemolysis finally attained, the only satisfactory method of studying their effect on the rate

of hemolysis as such would be to work with very strongly hypotonic solutions, or preferably, with distilled water. Most methods for studying hemolysis are much too slow for use in experiments of this type—which doubtless accounts for the fact that they have apparently not heretofore been made. The method of one of the authors (Jacobs, 1930) is entirely suitable for this purpose, however, and some results obtained with it may now be described.

In Table III are indicated the effects on hemolysis by water of the addition of different amounts of ethyl, n-butyl, i-amyl and phenyl urethanes. Though for such short times the experimental errors are

TABLE III

Effect of Various Urethanes on the Time Required for 75 Per Cent Hemolysis of Ox Blood by Distilled Water

Ethyl Urethane		n-Butyl Urethane		i-Amyl Urethane		Phenyl Urethane *	
Concentration	Time	Concentration	Time	Concentration	Time	Concentration	Time
	seconds		*seconds*		*seconds*		*seconds*
—	1.5	—	1.3	—	1.30	—	1.25
0.0313	1.5	0.0015	1.4	0.0003	1.30	0.00025	1.25
0.0625	1.5	0.0031	1.4	0.0013	1.28	0.0005	1.30
0.125	1.7	0.0062	1.5	0.0025	1.32	0.001	1.30
0.25	2.0	0.0125	1.6	0.005	1.42	0.002	1.35
0.5	2.2	0.025	1.5	0.01	1.50	0.004	1.32
1.0	2.6	0.05	2.1			0.008	1.52

* See also Table V. .

relatively large, it will be noted that the results are on the whole entirely consistent and that for each substance there is a slight but unmistakable retardation of hemolysis as the concentration increases. Furthermore, the effectiveness of the different urethanes, as might be expected, proves to be very different. As judged by the dilutions at which a retardation of hemolysis first becomes apparent, the order of effectiveness is:

$$\text{ethyl} < \text{n-butyl} < \text{i-amyl} < \text{phenyl}.$$

This is not only the order in which narcotic effects are usually manifested by these substances, but there is a rough quantitative agreement between the concentrations at which hemolysis begins to be retarded and those found by Dr. E. B. Harvey to be effective in reversibly suppressing the cleavage of the *Arbacia* egg. The latter concentrations, as cited by Lucké (1931), are as follows: ethyl, 0.05 M to 0.2 M; n-butyl, 0.0125 M to 0.05 M; i-amyl, 0.005 M to 0.01 M; and phenyl, 0.00125 M to 0.005 M.

In the case of ethyl urethane, where the effective concentration is fairly high, it is conceivable that at least a part of the delay in hemolysis may be due to osmotic factors. Even a substance that penetrates a cell as rapidly as a urethane might, if sufficiently concentrated, slow to a measurable extent the rate of attainment of the final osmotic equilibrium between the cell and its surroundings, while having no direct effect upon the position of the equilibrium. Whatever may be the validity of this objection in the case of ethyl urethane, however, it is certain that it cannot hold in the case of the other three substances, where the effective concentrations are of the order of 0.01 M to 0.001 M. It has been shown elsewhere by one of the authors (Jacobs, 1932) that even in the case of a completely non-penetrating non-electrolyte such as saccharose, the time required for hemolysis in a 0.01 M solution differs from that in

TABLE IV

Times required for 75 per cent hemolysis of ox blood in NaCl solutions containing different amounts of ethyl urethane. Temperature, 20° C.

Concentration of Urethane	Concentration of NaCl							
	Water	0.02 M	0.04 M	0.06 M	0.07 M	0.073 M	0.076 M	0.08 M
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
——	1.5	4.0	5.7	7.0	10.3	18.0	22.5	70.2
0.0313 M.....	1.5	4.2	5.6	7.5	11.7	19.8	26.8	73.4
0.0625 M.....	1.5	4.0	5.8	7.3	12.5	18.9	34.4	96.5
0.125 M.....	1.7	4.0	6.2	8.3	16.0	27.8	56.5	156
0.25 M.....	2.0	4.7	6.5	9.1	20.3	54	103.5	332
0.5 M.....	2.2	4.8	7.0	9.7	39.0	140	280	832
1.0 M.....	2.6	5.1	7.1	10.3	32.6?	274	317	420

water only to a barely measurable extent, the difference for ox erythrocytes being perhaps 0.1 second. With lower concentrations than 0.01 M of substances of extremely high penetrating power such as the urethanes, direct osmotic effects could certainly not be measured; and any effects that could be measured would therefore necessarily be of a more specific nature. Though the retardation of hemolysis in water by urethanes is apparently always small in amount and the errors of the determinations are relatively large, the results obtained in these and in other experiments have been sufficiently consistent to leave little doubt that the substances in question are able to affect the rate of hemolysis under conditions where any shift in the final theoretical equilibrium is of negligible importance. Such an effect, though erroneously inferred by other workers from their experiments, has not, we believe, previously been demonstrated.

In concluding the presentation of experimental data there may be added in Tables IV and V figures showing the gradual transition from conditions where the fundamental rate factor is primarily concerned in determining the time of hemolysis to those where the equilibrium factor tends to dominate the situation. In the light of the facts presented in the preceding section, it is evident that the striking effects produced by all the substances in the most concentrated salt solutions are due chiefly to a change of the hemolytic end-point in the direction of a reduced final degree of hemolysis. The rate factor as such cannot be studied in such solutions. In the case of ethyl urethane, one additional factor appears in the last figure of the last column, namely, a direct hemolytic effect of

TABLE V

Times required for 75 per cent hemolysis of ox blood in NaCl solutions containing different amounts of phenyl urethane. Temperature, 20° C. Each figure for water and for the lower concentrations of NaCl is the average of four determinations.

Concentration of Urethane	Concentration of NaCl						
	Water	0.02 M	0.04 M	0.06 M	0.075 M	0.08 M	0.085 M
	seconds	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*	*seconds*
——	1.50	3.87	5.17	6.92	11.1	19.8	41
0.0005 M	1.50	—	5.17	6.70	11.8	28.5	46
0.001 M	1.55	3.88	5.23	6.72	12.8	30.0	55
0.002 M	1.52	3.85	5.20	7.05	14.5	76.5	115
0.004 M	1.70	3.90	5.30	7.58	48.5	86.0	—
0.008 M	1.95	3.93	5.38	7.95	54.2	121.0	870

the urethane in very high concentrations; but this factor is of comparatively small importance in connection with the present experiments.

One other point suggested by the results obtained with i-amyl and phenyl urethanes may be mentioned briefly, namely, that whereas the retarding effect of urethanes on hemolysis by water never failed to appear in our experiments, it was sometimes more doubtful in the case of the most dilute salt solutions, *e.g.*, 0.02 M. The experiment chosen for representation in Table V was selected to show this condition, which was, however, not invariably observed. We are unable to suggest a reason why the retarding effect of certain urethanes may at times be more pronounced in the absence than in the presence of salts, but may point out that a somewhat similar but much more striking effect has been observed by Lucké (1931) in the case of the *Arbacia* egg. His explanation is that if the entrance of water into the cell has already been strongly retarded by the presence of salts (especially those of calcium), narcotics are unable to produce any further effect. In the case of the

erythrocyte it will be shown in a later paper that very low concentrations of salts, including NaCl, are effective in some non-osmotic manner in retarding osmotic hemolysis. It is conceivable, therefore, though by no means certain, that a similar principle is involved in the two cases.

IV

It has been shown in the preceding sections that various urethanes in the proper concentrations are able to reduce the degree of hemolysis finally attained in certain hypotonic salt solutions. This increase in the osmotic resistance of the cells, which has been noted by a number of previous observers, is an equilibrium rather than a rate effect and is best seen in solutions whose concentrations are such as to cause the disappearance of some but not all of the erythrocytes in the given sample of blood. It has also been shown that under conditions where complete hemolysis is very rapidly produced and where possible changes in the theoretical position of equilibrium of the system are of negligible importance, narcotics are able to bring about a slight but consistent slowing of the rate of the process. This effect, which is best seen in distilled water and which has frequently been confused with the one first mentioned, has not as far as we are aware, previously been demonstrated, though from the standpoint of cell permeability it is the more important of the two. Its possible nature will be considered after attention has first been given to the more striking and better known change in the osmotic resistance of the cells.

As has been mentioned above, there are at least four different ways in which the osmotic resistance of the erythrocyte might be affected (factors *a*, *b*, *c* and *d* on page 314). Of these factors, the first, namely, a change in permeability to water, may almost certainly be ruled out as a possible cause of any change in the position of final equilibrium of the hemolytic system. Only if the cells at some point became completely impermeable to water could this factor alone do more than change the rate at which the equilibrium is reached; the equilibrium itself would remain unaltered. As a matter of fact there is an abundance of evidence that the erythrocytes do not at any time become completely impermeable to water.

On the other hand, a changed permeability to salts and other osmotically active substances contained within the cell (factor *c*) might conceivably alter its osmotic resistance. Since hemolysis by hypotonic solutions is due to an excess of osmotic pressure within the cell, any escape of materials that reduced this excess would not only slow the rate of hemolysis in all cases, but in certain critical cases would prevent its occurrence altogether. The possibility of a leakage of salts from the

erythrocyte in ordinary osmotic experiments has been emphasized by Ponder and Saslow (1931), and it is by no means inconceivable, or indeed unlikely, on *a priori* grounds that such a leakage might be favored by narcotics. In sufficiently high concentrations these substances tend by destroying the erythrocyte to permit a very ready escape of materials from its interior; and it is entirely possible that in lower concentrations they might injure its surface sufficiently to increase any loss of electrolytes already in progress.

Though this theory has a certain degree of plausibility, it nevertheless seems necessary to discard it in view of the direct evidence obtained by Siebeck (1922) that narcotics actually reduce to an easily measurable extent the rate of exchange of ions between the cell and its surroundings, and that furnished by Joel (1915) that the gradual increase in the electrical conductance of a suspension of erythrocytes is slowed rather than accelerated in the presence of such substances. As far as the available evidence goes, the effect of ordinary concentrations of narcotics would seem, if this factor were of importance, to be in the direction of reducing rather than of increasing the osmotic resistance of the erythrocyte.

Turning next to factor *d*, which involves some change or changes in the osmotic resistance of the cell not associated with permeability factors, the possibility suggested by Traube that the narcotic may produce in some way a thickening or a strengthening of the cell membrane and so oppose hemolysis may first be considered. Such an explanation appears to be an unlikely one in view of the fact that the surface of the erythrocyte seems normally to offer little resistance to osmotic volume changes. (See in this connection Jacobs 1931, 1932.) As a matter of fact, the increased resistance in the presence of, for example, 0.3 M ethyl urethane, which is by no means the greatest effect we have observed, may correspond to a change in the critical concentration of NaCl by 0.003 M, amounting in terms of osmotic pressure to perhaps one-eighth of an atmosphere. That the delicate cell membrane could be strengthened to support this excess of pressure does not seem very likely.

A much more plausible possibility is that the narcotic may in some way have a tendency to cause a diminution in the volume of the cell and so to oppose its swelling in hypotonic solutions. Effects of this sort are already known in the case of other agents. For example, the increased osmotic resistance of erythrocytes in alkaline media and at high temperatures (Jacobs and Parpart, 1931) and in solutions of non-electrolytes (Jacobs, 1932) is probably to be accounted for in this way. As a matter of fact, v. Knaffl-Lenz (1918) has reported a decrease in the volumes of erythrocytes, as measured by the hematokrit, on the addition

of certain narcotics, though the times required to produce this effect in his experiments were much longer than those involved in the present series; and, furthermore, his results were indecisive in the case of the only urethane he used. We have been unable to detect with certainty by the hematokrit method any such differences in volume in the case of ethyl urethane solutions, though in view of the rather large errors of the hematokrit method and the very slight volume changes required to produce a considerable difference in the observed percentage of hemolysis (Jacobs and Parpart, 1931) we do not feel that this possibility has been entirely ruled out.

The last factor that will be discussed is the second of those mentioned above, namely, the escape of hemoglobin from the cell. This factor has frequently been neglected in studies on osmotic hemolysis in the past owing, no doubt, to the old belief that hemolysis is produced by an actual bursting of the cell when the internal pressure has reached a sufficiently high point. If this were the mechanism of hemolysis, then the escape of hemoglobin would, in fact, be an unimportant part of the process. It seems certain, however, from the phenomenon of "reversible hemolysis," so-called, that the cell is not ordinarily ruptured by mild hemolytic agents, but that at a certain time, as a result of stretching or some other change in its surrounding membrane, the latter becomes permeable to the hemoglobin contained within the cell. This permeability to hemoglobin is reached in such a sudden and definite manner that osmotic hemolysis is apparently an "all-or-none" phenomenon, *i.e.*, up to a certain point no hemoglobin escapes from the cell; beyond that point an almost infinitesimal increase in the volume of the cell results in the free outward diffusion of all of its hemoglobin (Saslow, 1929; Parpart, 1931). Unfortunately, we know too little at present about the physical state of the hemoglobin within the cell and the possible effects of changes in this state on its diffusibility. It is usually assumed, however, in the absence of evidence to the contrary, that under all usual conditions we have to do with a simple aqueous solution of hemoglobin and that the possibility of its escape from the cell depends merely on the character of the cell membrane.

The assumption that the escape of hemoglobin from the erythrocyte depends primarily on the cell membrane may or may not be true. It is of interest, however, to see whether it can be made the basis of a plausible explanation of the effect of narcotics on osmotic resistance. There is considerable evidence that at the surface of the erythrocyte in addition to lipoid substances which give to the cell certain of its physical properties (Mudd, S., and E. B. H. Mudd, 1926) and which perhaps determine its free permeability to all lipoid-soluble substances, there are

regions through which water, ions, and non-lipoid-soluble organic substances of low molecular weight can pass. Though the exact structural nature of these regions is not known, they may, at least in a semi-figurative sense, be called " pores." A further discussion of this theory as applied to the erythrocyte is given by Mond and Hoffman (1928) and by Jacobs (1931).

Whatever may be our ideas of the exact nature of the hypothetical " pores " in the cell membrane, it must not be forgotten that certain purely objective facts are well known; namely, that non-lipoid-soluble molecules of sufficiently low molecular weight pass through the wall of the erythrocyte readily, those of higher molecular weight more slowly, and those whose molecular weight (or molecular volume) exceeds a certain size fail to do so at all. The hemoglobin molecule, of course, enormously exceeds the critical size for penetration. Nevertheless, in osmotic hemolysis a point is somewhere reached where rather suddenly the cell becomes permeable to hemoglobin. Without attaching too literal a meaning to the statement, we may say that at this point the " pores " have been enlarged sufficiently to permit the escape of this molecule.

Now we have a certain amount of experimental evidence that narcotics are able—presumably by adsorption—to diminish the size of the pores in artificial membranes, or, at any rate, to render more difficult the passage of certain substances through these membranes (Anselmino, 1928 *a, b*). Suppose that the same were true of the erythrocyte at the point where it undergoes hemolysis. In this case, the presence of a sufficient concentration of a narcotic substance might be expected to convert a " pore " that would otherwise just permit the passage of hemoglobin into one that would just fail to permit it. Further swelling would be necessary to cause hemolysis. The osmotic resistance of the cell would thereby be raised, just as it is known to be in fact. Furthermore, the effectiveness of weakly adsorbed narcotics would be less than that of strongly adsorbed ones and, again, there is a parallel between the adsorbability of different urethanes and their ability to prevent hemolysis.

Accepting in a purely tentative manner this explanation of the effect of narcotics upon the final equilibrium of a hemolytic system, how would such an explanation fit the known facts concerning the rate at which hemolysis occurs in very strongly hypotonic solutions? It is entirely conceivable that in such solutions the rate of osmotic hemolysis might be affected either by a slowing of the rate of entrance of water or by a slowing,—though not a prevention—of the escape of hemoglobin by a delay in the attainment of the proper condition of the pores, or by a combination of both factors. Since it is unlikely, with a rate of increase of cell volume as rapid as that in distilled water, that the delay

in the escape of hemoglobin would be very great, it seems entirely possible that at least a part of the observed effect of narcotics on the rate of hemolysis by water may be due to an actually decreased rate of penetration of this substance. It is to be noted, however, that the possible effect must in any case be rather slight.

In this connection it is of interest to consider the work of Siebeck (1922) on the effect of narcotics on the rate of passage of ions between the erythrocyte and its surroundings and also that of Anselmino and Hoenig (1930) on the entrance of the non-electrolytes erythritol, arabinose, xylose, etc. In the former case, actual chemical analyses were made at several intervals and it is therefore virtually certain that the permeability of the cell to the substances in question was dealt with directly. In the work of Anselmino and Hoenig, though the methods were not quite so direct, it is also very likely that their interpretation of their results as indicating a production by narcotics of a decreased permeability to various slowly penetrating non-electrolytes is correct. It is perhaps significant that the decrease in permeability to ions and to rather slowly penetrating non-electrolytes is much greater than any decrease for water that could be inferred from the present experiments. If the "pore" theory were correct, it would be expected that the hypothetical diminution of the pore diameter produced by narcotics would exert an effect upon permeability which would become proportionately greater as the size of the molecule increased. The water molecule, being the smallest of those commonly supposed to enter the erythrocyte in this manner, would be affected least of all.

It should be emphasized that this explanation of the manner in which narcotics may conceivably affect osmotic hemolysis is suggested merely as a convenient working hypothesis. Its chief advantages are that it explains in essentially the same manner both "rate" and "equilibrium" effects and that, as far as we are aware, it is not incompatible with any known facts. It is by no means necessary, however, that the rate and equilibrium effects should be explained in the same way; in the case of temperature, for example (Jacobs, 1928), they seem almost certainly to be of a different nature. It is entirely possible that at any time facts may come to light with which the present theory is inconsistent; in that case it may readily be abandoned without greatly changing the significance of the experimental data here presented.

We are glad to acknowledge our indebtedness to Dr. Balduin Lucké for supplying most of the urethanes used in this work, and to Ethel R. Parpart and G. E. Shattuck for assistance in connection with several of the experiments.

Summary

1. The observation of previous investigators that narcotic substances in proper concentrations tend to oppose osmotic hemolysis is confirmed in the case of several urethanes.

2. It is shown that the conclusion frequently drawn from such observations, that the antihemolytic effect of narcotics is due to a decreased permeability of the erythrocyte to water, is unwarranted by the existing experimental evidence. The necessity for a separation of "rate" and "equilibrium" factors in studies on osmotic hemolysis is emphasized.

3. It is shown by experiments in which these factors are properly separated that a slight but measurable retardation of osmotic hemolysis may be produced by low concentrations of urethanes. The possible nature of the mechanism of this retardation, which may perhaps in part involve a decreased permeability of the cell to water, is discussed.

BIBLIOGRAPHY

ANSELMINO, K. J., 1928*a*. *Pflüger's Arch.*, **220**: 524.

ANSELMINO, K. J., 1928*b*. *Biochem. Zeitschr.*, **192**: 390.

ANSELMINO, K. J., AND E. HOENIG, 1930. *Pflüger's Arch.*, **225**: 56.

ARRHENIUS, S., AND F. BUBANOVIĆ, 1913. *Meddel. från K. Vet-Akad.* Nobelinstitut, **2**: No. 32, p. 1.

JACOBS, M. H., 1928. *Am. Nat.*, **62**: 289.

——— 1930. *Biol. Bull.*, **58**: 104.

——— 1931. *Ergebn. d. Biol.*, **7**: 1.

——— 1932. *Biol. Bull.*, **62**: 178.

JACOBS, M. H., AND A. K. PARPART, 1931. *Biol. Bull.*, **60**: 95.

JARISCH, A., 1921. *Pflüger's Arch.*, **186**: 299.

JOEL, A., 1915. *Pflüger's Arch.*, **161**: 5.

VON KNAFFL-LENZ, E., 1918. *Pflüger's Arch.*, **171**: 51.

LUCKÉ, B., 1931. *Biol. Bull.*, **60**: 72.

MOND, R., AND F. HOFFMANN, 1928. *Pflüger's Arch.*, **219**: 467.

MUDD, S., AND B. H. MUDD, 1926. *Jour. Exper. Med.*, **43**: 127.

PARPART, A. K., 1931. *Biol. Bull.*, **61**: 500.

PONDER, E., AND G. SASLOW, 1931. *Jour. Physiol.*, **73**: 267.

SASLOW, G., 1929. *Quart. Jour. Exper. Physiol.*, **19**: 329.

SIEBECK, R., 1922. *Arch. f. exper. Pathol.*, **95**: 93.

TRAUBE, J., 1908. *Biochem. Zeitschr.*, **10**: 371.

YOSHITOMI, T., 1920. *Acta. Schol. Med. Univ. Imp. Kioto*, **3**: 338.

THE LIFE HISTORY OF PARORCHIS AVITUS (LINTON) A TREMATODE FROM THE CLOACA OF THE GULL

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The study of parasitic flatworms in the United States began with Joseph Leidy, who described a number of larval and adult trematodes. For many years progress consisted in the description of new species of adult flukes. More recently, attention has been directed to a study of the larval stages and at present one hundred and sixty-six cercariæ have been described in the United States. Only twenty of these larvæ, however, have been definitely correlated with their adult forms. It is obvious therefore that further advance is dependent upon knowledge of the life history of these species. Lack of such information keeps the literature burdened with almost twice as many specific names as are necessary, since each larval trematode whose adult stage is not recognized, is described as a distinct species. Furthermore, and of more significance, knowledge of the successive stages in development is indispensable for correct identification, taxonomy, and any fundamental work on the physiology and control of these parasites. The fact that fifteen of the twenty life histories solved in this country have been described in the last four years indicates the intensity of present interest in life history studies. This paper adds another to the list of known life histories and describes more completely the results reported by the writers in a recent preliminary note (Stunkard and Cable, 1931).

Experiments conducted at the Marine Biological Laboratory during the summer of 1931 have shown that *Cercaria sensifera* (Stunkard and Shaw, 1931) is the larva of *Parorchis avitus* (Linton, 1914). *Parorchis avitus* was originally described from the cloaca of the herring gull, *Larus argentatus*. It is very similar to *Parorchis acanthus*, a species described by Nicoll (1906) from the bursa and cloaca of *Larus argentatus* and first given the name *Zeugorchis acanthus*. Subsequently Nicoll (1907*a*) erected the genus *Parorchis* to supplant *Zeugorchis* (preoccupied) and named *P. acanthus* as type species.

Parorchis acanthus has been the subject of important studies by English investigators. Nicoll (1907*b*) gave a more complete description of the species and reported its occurrence in the common gull, *Larus canus*. In the same work he transferred *Distomum pittacium* Braun,

1902 to the genus *Parorchis*. Nicoll, in a letter to Linton, expressed the belief that *Parorchis avitus* is probably identical with *P. acanthus*. Linton (1928) however, showed that *P. avitus* differs from *P. acanthus* in the same respects that *P. acanthus* differs from *Parorchis* (*Distomum*) *pittacium*, namely, the ratio of sucker diameters and the extent of uterine convolution. Lebour (1914) compared young stages of *P. acanthus* with *Cercaria purpuræ* Lebour, 1907 from *Purpura lapillus*, and concluded that this cercaria is the larval stage of *Parorchis acanthus*. She also emphasized the close affinity of the genus *Parorchis* to the genus *Echinostomum* and expressed the belief that in the life history of *P. acanthus* there must be a secondary intermediate host, probably a bivalve mollusk, as is the case with *Echinostomum secundum* (Lebour, 1908). In a later paper, Lebour and Elmhirst (1922) reported the encystment of cercariæ of *Parorchis acanthus*, together with the cercariæ of *Echinostomum secundum* in the foot and mantle of *Cardium edule* and *Mytilus edulis*. No feeding experiments were made in determining the life history of *P. acanthus*, and conclusions were based entirely on morphological comparisons.

Methods and Observations

The present study consisted of controlled feeding experiments, since this is the only conclusive method of determining the life history. The oyster drill, *Urosalpinx cinereus*, was used exclusively as a source of cercariæ since the study was practically completed before it was discovered that *Thais* (*Purpura*) *lapillus* also served as host for this species. The infected snails were isolated in finger bowls. Cercariæ escaped from them in large numbers and encysted on the bottom of the bowl. These cysts were scraped off with a scalpel and concentrated by decantation. Attempts were made to infest terns, guinea pigs, white rats, and mice. Although the natural host of *P. avitus* is the herring gull, *Larus argentatus*, for the experiments, nestlings of two species of terns, the common tern, *Sterna hirundo*, and the roseate tern, *Sterna dougalli*, were employed since they were more easily obtained. The newly hatched nestlings were taken from a tern rookery on Big Weepecket Island, near Woods Hole, and were kept alive in the laboratory as long as possible. These birds, which had received no food while in the nest, were fed the deep flesh of mackerel and flounders, since trematode cysts are not as abundant in these tissues as in the skin and flesh near the surface of the body. Fishes showing any signs of being parasitized were not used for food. The nestlings were kept in the laboratory for two days, or until they began to take food readily, before infestation was attempted. They were then fed large numbers of cysts by means of a pipette. The

first lot of 6 birds was fed cysts to determine whether infestation could be established. Five of these died at night and in all cases were so badly disintegrated by the following morning that the alimentary tract had almost completely disappeared. The sixth bird refused to eat on the tenth day and was killed. Twelve immature worms in various stages of development were recovered from the cloaca. The second lot consisted of eight birds. Six were fed cysts on the same day and two were kept as controls. The nestlings lived for varying lengths of time, two of the roseate species surviving as long as 15 days. Table I gives the results obtained with this series.

TABLE I

No.	Species	Fed Cysts	Autopsied	No. Worms
1	S. hirundo	July 11	July 16	11
2	" "	"	" 17	13
3	" "	control	" 17	none
4	" "	"	" 18	"
5	" "	July 11	" 20	3
*6	" "	"	" 21	none
7	" dougalli	"	" 26	13
8	" "	"	" 26	28

* This bird died during the night and was badly disintegrated when autopsied, which may explain the absence of worms.

It is seen that every bird which was fed cysts, and on which a favorable autopsy could be made, became infected, while the two controls were negative. The oldest worms were recovered from the two roseate terns which lived 15 days after infestation was established. Although not sexually mature, these specimens were sufficiently developed to be identified positively as *Parorchis avitus;* this identification has been confirmed by Professor Edwin Linton.

Further attempts at experimental infection were made, using mammals instead of birds. Four guinea pigs, nine rats and six mice were fed large numbers of encysted larvæ and were autopsied after varying lengths of time. No worms were recovered from any of these animals. Although the larvæ may possibly excyst in these mammals, it is evident that conditions are not suitable for establishing infection.

STAGES IN THE LIFE CYCLE

The sexually mature worms are viviparous and miracidia occur free in the terminal part of the uterus. Each miracidium contains a single, fully formed redia and there is no sporocyst generation in the life history. These stages were described by Linton (1914). The rediæ

(Figs. 1 and 2) and cercariæ (Fig. 3) were described by Stunkard and Shaw (1931). It should be recorded that in addition to *Urosalpinx cinereus, Thais* (*Purpura*) *lapillus* also has been found to serve as the intermediate host of *Parorchis avitus*. Of 108 specimens of *Thais lapillus* collected August 1st at Weepecket Island, 8 were found to be infected. The appearance of the parasitized gonad and digestive gland of this snail was similar to that reported for *Urosalpinx cinereus* (Stunkard and Shaw, 1931). Of 610 *Urosalpinx* examined during the summer of 1931, 25 were found to be infested. This determination is based on the emergence of cercariæ from the isolated snails. The infestation of *Thais,* however, was found by crushing and examining them.

Most of the cercariæ encyst within 48 hours after leaving the snail. Encystment may be accelerated by mechanical stimulation such as stirring or shaking. Concentrated solutions of vital dyes also induce cyst formation. Before encysting, the cercariæ attach themselves by means of the suckers to the bottom of the dish or to the slide as the case may be, and become comparatively quiet for a few moments. The cystogenous material is then exuded, enclosing the body in a viscous mass. This material adheres to the tail, pushing it off from the body as additional cystogenous material is extruded. The tail may remain attached to the cyst for some time or may break off as a result of its vigorous movement. Decaudation before encystment has been observed a few times but this is not the rule. In either case, the tail soon disintegrates. The cystogenous material is poured out very quickly and remains in a semi-liquid state for about a minute, as indicated by its yielding to the movement of the enclosed worm. In a few minutes, however, the cyst becomes hardened. The cyst wall (Figs. 4 and 5), which is flattened on the side of attachment, consists of two layers, an outer thick and brittle one, and an inner membrane which is thin and tough. The outer wall is often broken when the cysts are scraped from the bottom of the dish with a scalpel.

The larva becomes shrunken immediately if the inner cyst membrane is torn and the worm comes in contact with sea water. This interesting phenomenon seems to be due to the hypertonicity of the sea water, although it may be the result of mechanical injury. If the former is the case, encystment must involve an important change in the body of the worm which before encystment can live perfectly well in sea water. The cyst is fairly transparent and the larva can be clearly observed within it. The position of the larva in the cyst is shown in Figs. 4 and 5. The acetabulum is often forced to the side of the median plane because of its large size. A fluid fills the space between the larva and the cyst wall. It is clear and in some cases contains small granules which have

probably escaped from the excretory system of the worm. The main excretory tubules with their concretions are easily observed through the cyst wall. The larva moves for several hours after encystment and then becomes quiescent. Movement may be induced by warming very slightly; it ceases upon cooling. The encysted worms may remain alive for an extended period if kept in water, but they do not withstand any considerable desiccation. They have been made to move two weeks after encystment; it is not known how long they can survive.

Specimens of *Mytilus edulis* were placed in finger bowls containing hundreds of cercariæ in order to determine whether the larvæ would encyst within any part of the mollusk. These experiments were all negative. In one case, three cysts were found *on the surface* of the foot and mantle, and a few others were seen in masses of mucus in the mantle cavity. In the same specimen, large numbers of cercariæ encysted on the outside of the shell, most of them near the incurrent siphon. Many of them struck the shell around the incurrent opening, adhered, and immediately encysted. The significance of these results will be brought out in the discussion.

Since birds could be infected by feeding the encysted worms, and since the larvæ encyst on any available object, it is apparent that a second intermediate or transfer host is not an essential stage in the life cycle of the parasite. It is clear that the final hosts are infected by accidentally ingesting the larvæ with food on which they have encysted. The worms excyst in the intestine of the bird and develop to maturity in the cloacal region. In autopsies of experimentally infested birds, a few worms were found just behind the intestinal ceca, but none in the ceca themselves. Since sexually mature worms were not obtained, the following description is based on specimens recovered 15 days after infection. These worms possess all of the characteristic adult structures except the distended gonoducts. They are white in color and are seen only with difficulty on the wall of the cloaca. It is very difficult to remove the worms from the surface of the cloacal epithelium on account of the powerful suction of the acetabulum. After removal to Ringer-Locke's solution, specimens have been kept alive for twelve hours before fixation. It is not known how much longer they would have survived. If not removed from the cloaca soon after the death of the host, however, they die and disintegrate rapidly along with the tissue to which they adhere. The intestine of a bird is frequently disintegrated 6 to 8 hours after its death, which explains the negative results in the first series of experiments.

When the worms are placed in Ringer-Locke's solution, they move actively for about an hour. This movement is very similar to that of the

cercariæ. The worms, although flat and leaf-like, tend to lie on one side, *i.e.*, with the ventral surface in a vertical rather than a horizontal position, movement being effected by flexing and extending of the preacetabular region of the body. After this period of active movement during which the suckers are not used, the worms settle to the bottom of the dish, become attached by means of the suckers, and move in measuring worm fashion. This means of locomotion is more effective than the erratic dorso-ventral lashing of the forebody. Finally, however, the worms become quiescent on the bottom of the dish, remaining in some cases sufficiently still to be drawn with the camera lucida; when stimulated by moving the dish or touching them with a needle, they again swim actively for a time.

The morphology of *Parorchis avitus* was described by Linton (1914). Study of the present specimens confirms the observations of Linton and permits certain additions to the description of the species. The average measurements of thirteen 15-day worms stained and mounted are: length, 1.8 mm.; width of oral sucker, 0.236 mm.; width of pharynx, 0.1 mm.; and width of acetabulum 0.433 mm. The details of spination are shown in Fig. 6. There are about 68 spines in the collar. The papillæ, noted in the larvæ, still persist on the anterior end of the 15-day worm and are most clearly seen on a well extended but not flattened living specimen. The antero-ventral surface is the most heavily spined region of the body. The alternate rows of closely set spines extend backward in regular order to the genital ridge. Behind this region, there is a gradual thinning out on either side of and posterior to the acetabulum, with a few scattered spines extending to the extreme posterior end. The body is flattened dorsoventrally, especially in the postacetabular region, although with sexual maturity this portion will undoubtedly enlarge.

The excretory system, which is one of the most striking features of these specimens, differs markedly from the condition in the cercarial stage described by Stunkard and Shaw. In the cercaria there is an excretory vesicle at the caudal end of the body and collecting ducts pass forward on either side to the region between the pharynx and oral sucker where they turn posteriad. The recurrent ducts divide at the level of the intestinal bifurcation into anterior and posterior branches. Each of the anterior and posterior branches bears three groups of flame cells, with three cells in each group. In specimens removed from the cloaca of the tern the ascending collecting ducts of the cercaria have developed complicated series of evaginations which tend to form a reticulum and which ramify through the body of the worm. This ramification of the ascending ducts has not been observed in encysted larvæ and probably

does not appear before excystment. A number of very small worms were recovered from the tern that was fed cysts intermittently. The youngest of these, which had probably been excysted not more than two or three days, exhibited to a considerable degree the complex ramification of the main excretory trunks. In general, the excretory system agrees with that of *P. acanthus* as described by Nicoll (1907 *b*). The excretory vesicle is a conspicuous, irregularly shaped, transparent sac at the posterior end of the body, and empties through a dorsal excretory pore (Fig. 6). The main lateral excretory trunks extend from the antero-lateral regions of the vesicle and branch in a very complicated manner. The rami extend in three general directions, laterad, mediad, and anteriad. The median branches either end blindly or anastomose with corresponding branches of the opposite trunk. The anterior branches are, for the most part, continuations of the main trunk which extend around either side of the acetabulum, anastomosing in some cases, and sending a number of sinuses into the region just above the acetabulum. The lateral branches extend laterally and somewhat ventrally, connecting with a more or less continuous row of large sinuses which extends along either side of the body. Tiny branches extend from the sinuses to the very edges of the body. The two rows of lateral sinuses extend posteriorly and in some cases fuse, making a connection behind and below the excretory vesicle. Anteriorly, the anterior sinuses decrease in size and send branches into the tissue just dorsal to the oral sucker. On each side, at the level between the oral sucker and

EXPLANATION OF PLATE

FIG. 1. A young redia with an immature cercaria and several germ balls (42 ×).

FIG. 2. An older redia containing only mature cercariae, one of which is seen escaping through the birth pore (42 ×).

FIG. 3. A mature cercaria (155 ×).

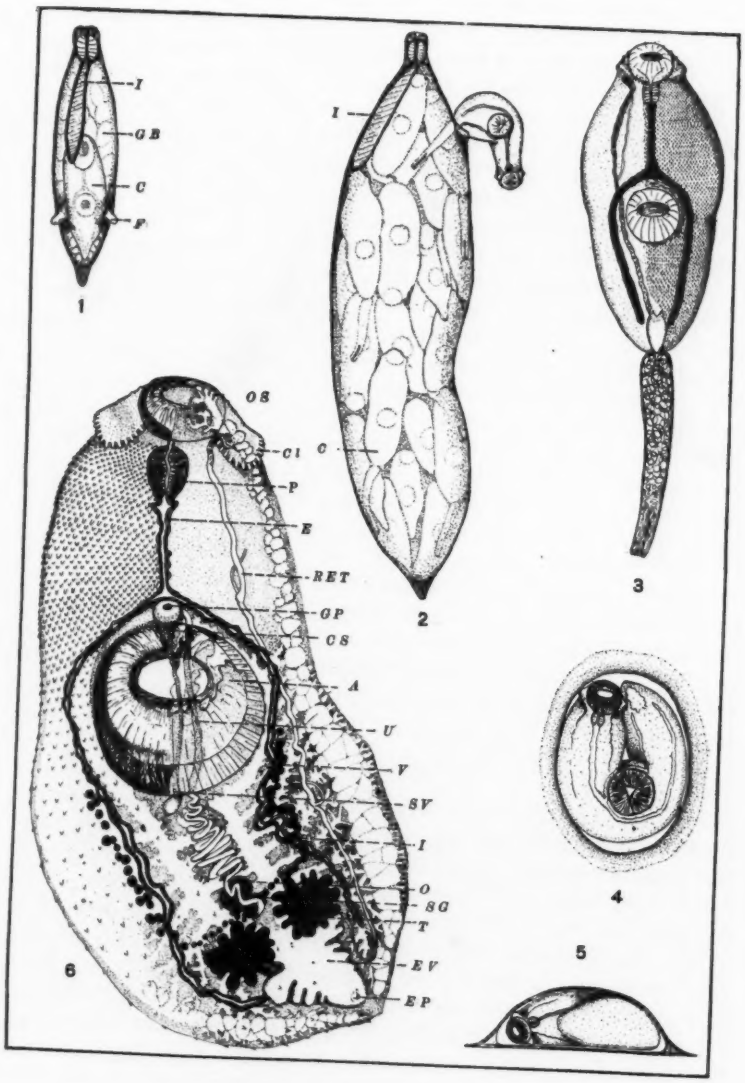
FIG. 4. An encysted cercaria, top view (132 ×).

FIG. 5. An encysted cercaria, side view (132 ×).

FIG. 6. Ventral view of a 15-day worm (60 ×).

LETTERING

A Acetabulum
C Cercaria
Cl Collar
CS Cirrus Sac
E Esophagus
EP Excretory Pore
EV Excretory Vesicle
F Foot
GB Germ Ball
GP Genital Pore
I Intestine
O Ovary
OS Oral Sucker
RET Recurrent Excretory Tubule
SG Shell Gland
SV Seminal Vesicle
T Testis
U Uterus
V Vitellaria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pharynx, the recurrent excretory tubules extend inward and backward from the lateral sinuses, dividing at the level of the intestinal bifurcation into anterior and posterior branches, as described in the cercaria. It is seen, then, in the adult that the complex sinus system as a whole corresponds to the main lateral trunks of the cercaria and, consequently, serves to connect the recurrent excretory tubules with the vesicle. The main tubules are ciliated and the entire system, with the exception of the recurrent tubules and their branches, contains numerous concretions. The movement of these excretory bodies in the sinuses is of assistance in tracing the connections of the system. The excretory fluid is forced through the spaces by movements of the worm, the direction of the flow depending on the movement. After living worms have been kept in Ringer-Locke's solution for some time, the sinuses and bladder collapse, expelling clouds of granules and considerable amounts of fluid through the excretory pore. This makes it necessary to study the excretory system in freshly removed material, if the precise relations of the vesicle and sinuses are to be observed. The worm may live, however, for many hours after these structures have become indistinct. The preacetabular region of the body also becomes opaque long before the worm dies, making observations on the finer details of the excretory system in this region more difficult.

The reproductive system (Fig. 6) of the 15-day worm is not fully developed, although in a few cases motile spermatozoa were observed in the seminal vesicle, and one case of copulation was noted. The uterus begins as a simple duct which extends forward from the oötype. As development proceeds it forms lateral convolutions, a process which continues as the worm becomes more mature and large numbers of eggs are produced.

Discussion

Since the close affinity of *Parorchis* and the echinostomes is apparent from morphological comparisons, and since the cercariæ of *Echinostomum secundum* were found by Lebour (1908) to encyst in bivalve mollusks, she expected the same behavior on the part of cercariæ of *P. acanthus*. Several facts, however, seem to indicate that Lebour and Elmhirst's (1922) conclusions in regard to the encystment of cercariæ of *P. acanthus* (*C. purpuræ*) in the mussel, *Mytilus edulis*, may be in error. Close relationship may not necessarily imply identical modes of life history. Johnson (1920) has shown in the case of *Echinostomum revolutum*, which is cogeneric with *E. secundum*, the cercariæ encyst both in the snail bearing the rediæ, and in turbellarians. Johnson emphasized the lack of specificity in this regard. Furthermore, Lebour

and Elmhirst's experiments were not adequately controlled. A criticism of their methods and results was made by Stunkard and Shaw (1931). The flattened side of the cyst, as figured by Lebour and Elmhirst, indicates that it had been formed on a surface over which it had spread somewhat before hardening, and not in the tissues of the foot or mantle. It is difficult to conceive the formation of a cyst of this shape within the soft tissue. The paucity of their positive results and the fact that their experiments were not sufficiently controlled, throw considerable doubt on the conclusions of Lebour and Elmhirst in regard to a second intermediate host for *P. acanthus*. Their negative results were explained by the assumption that the cercariæ were not ready for encystment, a certain amount of swimming being necessary before this process occurred.

In the present experiments, the cercariæ encysted quite readily on the shell of *Mytilus* but exhibited little or no tendency to do so on the foot or mantle. Johnson (1920) stated that larvæ of *Echinostomum revolutum*, encysted in the snail, were not killed by the death and disintegration of the host. The encysted cercariæ of *Parorchis avitus*, similarly, may not be adversely affected by the death and partial disintegration of organisms on which they encyst. The gulls, which are natural hosts of this trematode, are scavengers, feeding on dead material. It appears certain from observations made in the present study that the cercariæ, after leaving the snail, may encyst *on* almost anything with which they come in contact. The ease with which encystment can be induced by mechanical stimulation indicates that merely swimming against organisms, or even inanimate objects, may cause encystment. It has been shown that encystment and subsequent development in a secondary intermediate host are not necessary for the completion of the life history. The encystment of the cercariæ on anything that may become food for gulls provides their means of reaching the final host, and is the essential factor in completing the cycle.

A morphological comparison of *Parorchis acanthus* and *P. avitus* shows many similarities, particularly in regard to the excretory system, the reproductive organs, the scale-like spines of the collar and body, and the general body shape. A close comparison of the sizes of suckers and coils of the uterus is not possible at the present time since our specimens of *P. avitus* were not fully mature. Both the oral sucker and acetabulum increase rapidly in size after excystment, but the rate of increase is much greater in the case of the acetabulum, the ratio changing from about 1 : 1.4 in the cercaria to approximately 1 : 3 in the adult.

The similarities between the two species appear to be of generic significance, while present observations note additional specific differences between *P. acanthus* and *P. avitus*. The chief differences, com-

piled from the descriptions of Linton, of Lebour, and from the present study are: (1) the ratio of sucker diameters, which is 1:3 in *P.* avitus and 1:2 in *P. acanthus;* (2) the greater extent of uterine convolution in *P. avitus;* (3) *P. avitus* is viviparous whereas *P. acanthus* is oviparous; (4) the miracidia are very different in appearance; (5) the excretory system extends into the tail of the cercaria of *P. avitus,* whereas it does not enter the tail of *Cercaria purpuræ;* (6) the arrangement of the gland cells in the cercariæ is different; and (7) the larvæ of *P. avitus* do not encyst in the foot or mantle of *Mytilus edulis* as has been reported for *P. acanthus.*

Summary

The life history of *Parorchis avitus* has been experimentally traced. The cercariæ occur in the marine snails, *Urosalpinx cinereus* and *Thais* (*Purpura*) *lapillus.* Adults have been obtained from the cloaca of the common tern, *Sterna hirundo,* and the roseate tern, *Sterna dougalli,* after feeding the young birds with encysted larvæ.

It has been shown that a specific secondary intermediate host is not essential for the completion of the life history; only a means of transference is necessary.

Additional morphological differences between *Parorchis avitus* and *Parorchis acanthus* are described.

BIBLIOGRAPHY

Johnson, J. C., 1920. The Life History of Echinostomum revolutum (Froelich). *Univ. Calif. Publ. Zool.,* **19:** 335.

Lebour, M. V., 1908. A Contribution to the Life History of Echinostomum secundum (Nicoll). *Parasit.,* **1:** 352.

Lebour, M. V., 1914. Some Larval Trematodes from Millport. *Parasit.,* **7:** 1.

Lebour, M. V., and R. Elmhirst, 1922. A Contribution towards the Life History of Parorchis acanthus (Nicoll), a Trematode in the Herring Gull. *Jour. Mar. Biol. Ass'n., Plymouth,* N.S., **12:** 829.

Linton, E., 1914. Notes on a Viviparous Distome. *Proc. U. S. Nat. Mus.,* **46:** 551.

Linton, E., 1928. Notes on Trematode Parasites of Birds. *Proc. U. S. Nat. Mus.,* **73:** 1.

Nicoll, W., 1906. Some New and Little-known Trematodes. *Ann. Mag. Nat. Hist.,* (7) **17:** 513.

Nicoll, W., 1907*a*. Observations on the Trematode Parasites of British Birds. *Ann. Mag. Nat. Hist.,* (7) **20:** 245.

Nicoll, W., 1907*b*. Parorchis acanthus, the Type of a New Genus of Trematodes. *Quart. Jour. Micros. Sci.,* **51:** 345.

Stunkard, H. W., and R. M. Cable, 1931. A Trematode from the Cloaca of the Gull. *Science,* N.S., **74:** 438.

Stunkard, H. W., and C. R. Shaw, 1931. The Effect of Dilution of Sea Water on the Activity and Longevity of Certain Marine Cercariæ, with Descriptions of Two New Species. *Biol. Bull.,* **61:** 242.

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